# University of Cincinnati

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**I, Norah AlMulhem , hereby submit this original work as part of the requirements for the degree of Master of Science in Transfusion and Transplantation Sciences.**

It is entitled:

**Cryopreservation and Hypothermal Storage of Hematopoietic Stem Cells**

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# Cryopreservation and Hypothermal Storage of Hematopoietic Stem Cells

A thesis submitted to the Division of Research and Advanced Studies of the University of Cincinnati in partial fulfillment of the requirements for a degree of

MASTER OF SCIENCE

In the department of Analytical and Diagnostic Sciences of the College of Allied Health Sciences and the University of Cincinnati Academic Health Center

Hoxworth Blood Center

By

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**ABSTRACT**

The recent availability of commercially available storage media (CryoStor™ and HypoThermosol™) designed for optimal long-term and short-term hematopoietic stem cell (HSC) storage prompted an evaluation of hematopoietic stem cell and hematopoietic progenitor cell (HSC/P) viability and functionality after storage in these media formulations, compared with the conventional media used at Hoxworth Blood Center. Three human umbilical cord blood units (CBUs) were cryopreserved in CryoStor5 (CS5), CryoStor10 (CS10), and a conventional internally prepared cryopreservation medium, then analyzed post-thaw for viability and recovery of several mature and immature hematopoietic cell types, as well as for clonogenic capacity, and proliferation potential. Flow cytometric analysis indicated similar post-thaw viability of most cell subsets cryopreserved in CS5, and CS10 compared to the conventional cryopreservation medium (containing 5 % Dimethylsulfoxide (DMSO) and 2.5 % hydroxyethyl starch). This variation in viability was not statistically significant (p-value 0.2-1). Bromodeoxyuridine (BrdU) uptake was used to measure the ability of the frozen/thawed cells to proliferate in culture for 48 h in response to stem cell factor (SCF), FLt-3 ligand (FLt-3) and thrombopoietin (TPO). Proliferation potential and clonogenic capacity were both slightly better with after freezing in CS10; however, the differences were not statistically significant. This study shows that the conventional medium for cryopreservation used in our laboratory is similarly effective, compared with CS5 or CS10 media, in protecting the cryopreserved CBU derived HSC/P products.

The same analytical methods were used to compare HypoThermosol® (HTR-FRS®), which is designed for short-term refrigerated storage of hematopoietic cells, to a locally prepared

medium containing Plasma-Lyte A and 0.5 % human serum albumin (HSA). Measurements were performed after 24, 48 and 72 h of cold storage (4°C). Results showed similar viability and recovery after 24 h of storage, but after 48 and 72 h, a significant decline in viability occurred in a few of the subsets when stored in Plasma-Lyte A/HSA medium, compared to when stored in HTS-FRS®. Differences in clonogenic capacity and proliferation potential were not significant, however the cells' proliferation potential was slightly better after storage in HTS-FSR®. Taken together, these results indicate that the HTS-FRS® storage media preserves hematopoietic cell function better than Plasma-Lyte A/ 0.5 % HSA, especially if the cells are to be stored for more than 24 hours.

It is possible that these in-vitro results could translate to improved engraftment after storing umbilical cord blood, bone marrow or mobilized peripheral blood in these new media.

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## ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| ACD-A | Anticoagulant citrate dextrose formula A |
| $ALD^{br}$ | $ALDEFLUOR^{bright}$ |
| ALDH | Aldehyde dehydrogenase |
| APC | Allophycocyanin |
| APC-Cy7 | Allophycocyanin with cyanin-7 |
| Apo | Apoptosis |
| BM | Bone marrow |
| BrdU | Bromodeoxyuridine |
| BSC | Biological safety cabinet |
| CBU | Cord blood unit |
| Cont. | Control |
| CPA | Cryoprotective agents |
| CS | CryoStor |
| DMSO | Dimethylsulfoxide |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| FSC-A | Forward scatter (area) |
| HES | Hydroxyethyl starch (hetastarch) |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HLA | Human leukocyte antigen |
| HSA | Human serum albumin |
| HSC | Hematopoietic stem cell |
| HSC/P | Hematopoietic stem cell and progenitor cell |

| | |
|---|---|
| HTS-FRS® | Transport medium HypoThermosol-FRS® |
| IMDM | Iscove's Modified Dulbecco's Media |
| LCP | Lineage committed progenitors |
| MPP | Multipotent progenitors |
| NC | Nucleated cells |
| NMDP | National Marrow Donor Program |
| PE | Phycoerythrin |
| PrepaCyte | PrepaCyte CB and PrepaCyte WBC |
| RT | Room temperature |
| SB | Staining buffer |
| SCF | Stem cell factor |
| SSC | Side scatter (area) |
| TM | Thawing medium |
| TNC | Total nucleated cells |
| TPO | Thrombopoietin |
| µL | Microliter |
| 7-AAD | 7-Aminoactinomycin D |

# INTRODUCTION

## Hematopoietic stem cell transplantation

Stem cell transplantation is a promising treatment for many diseases. In current medical practice, it has mostly been used as a treatment for hematological diseases to reconstitute damaged bone marrow after aggressive treatment, such as chemotherapy or radiation therapy, or to replace non-functional marrow in bone marrow failure syndromes such as Fanconi anemia. Hematopoietic stem and progenitor cells (HSC/P) can be harvested from bone marrow (BM), cord blood and peripheral blood (after mobilization).

Human bone marrow transplantation started in 1959, when professor Mathé transplanted several patients after an accidental radiation exposure. This approach was based on a previous finding that demonstrated the ability of bone marrow cells to reconstitute damaged bone marrow.[2] The initial outcomes of BM transplantation were disappointing. In 1970, Bortin reported survival of only 3 out of 203 patients at 2 to 12 years period post transplantation.[3] Dausset's[4] and Van Rood's[5] discovery of the human leukocyte antigen (HLA) system opened doors for revolutionary improvements in hematopoietic stem transplantation as it allowed selection of donors based on HLA typing, and by 1973 the first unrelated BM transplant was done. Subsequently, other sources of HSC/P were found as the first mobilized peripheral blood transplant was in 1986[6], followed by the introduction of cord blood (CB) in 1988.[7]

The availability of multiple sources of HSC/P and the large HLA phenotype databases have enabled the establishment of national and international donation programs, such as the National Marrow Donor program (NMDP) and World Marrow Donor Association (WMDA). These organizations help to match the HLA type of the patients with national or

international donors. The NMDP data showed that the mobilized peripheral blood and CB have started to replace BM as a source of HSC that can be used in a clinical transplant practice.[8] The CB has a benefit over the BM and the mobilized peripheral blood, as it is easy to be collected, does not require donor drug treatments prior to collection and it is readily available. That makes it easier to be used in research than the earlier two.

**Hematopoiesis and hematopoietic stem cell subsets**

The hematopoietic stem cell's (HSC) self-renewal and multi-lineage differentiation abilities are the most important characteristics that help in the restoration of hematopoietic function in patients following transplantation. Hematopoietic stem cells differentiate into multipotent progenitors (MPP), and then to lineage committed progenitors (LCP), which will give rise to different blood cell types. Studies that used single-cell isolation proved that HSC are responsible for the long-term engraftment that lasts for 20 weeks or more. Engraftment of MPP cells peaks between 2-4 weeks then declines; therefore is considered as short-term engraftment.[9] Hematopoietic stem cells, progenitor and mature cells express different surface antigens that vary in nature and function. Flow cytometric analysis using specific fluorescent-labeled monoclonal antibodies against these surface antigens allows the quantification of various cell subsets (Figure 1).

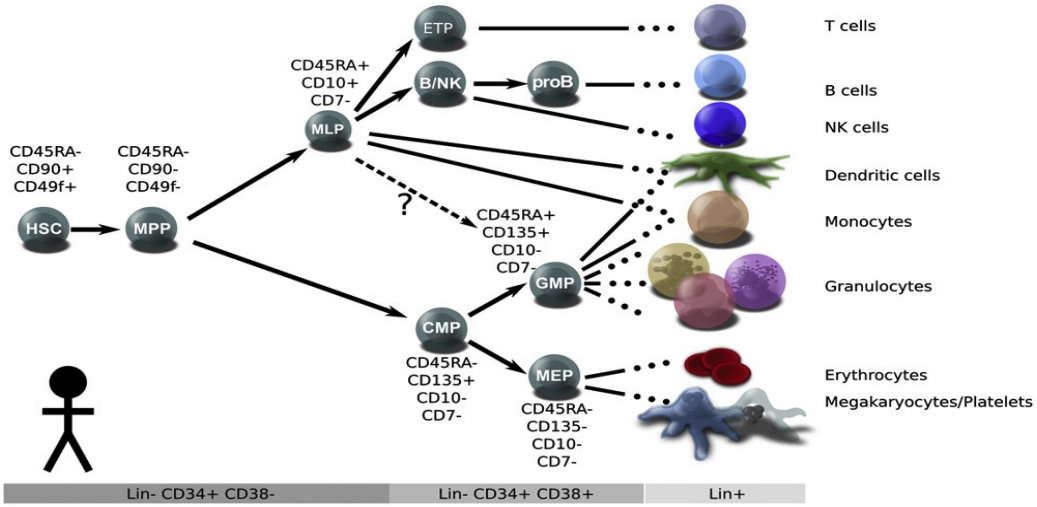



**Figure 1: Hematopoiesis.** [10]

In addition to cell surface antigens, the stem cell can be defined by biological characteristics such as its enzyme content. Stem cells contain higher levels of the enzyme aldehyde dehydrogenase (ALDH) than more mature cells , thus ALDH content can be used to measure stem cell content. [11]

In current clinical practice, the number of CD45+/CD34+ cells in the graft is used to determine the number of progenitor cells, and to calculate the cell dose available for transplantation. The CD34+ population is a heterogeneous population that is composed of multiple cell types that vary in stages of maturity and ability to proliferate and/or differentiate into various hematopoietic lineages. This heterogeneous cell population can either lose or gain the expression of surface antigens such as CD38 and CD90 along with differentiation. The most immature (stem cell-like) cell subset within the CD34+ population is the enriched HSC, which is CD34+, CD38- and CD90+ (abbreviated CD90+). As the CD90+ cells undergo differentiation, they lose CD90 expression, but are still capable of multilineage differentiation, and are thus called multipotent progenitor cells (MPP) (abbreviated CD90-). With continued differentiation, the MPP begin to become more lineage restricted and start to express the CD38 antigen, thus are named lineage committed progenitors (LCP)

(abbreviated CD38+). These progenitor cell populations are responsible for the early and late engraftment activities of the transplanted graft. There are other cell surface antigens that have been used to enrich for the HSC, as previous studies proved that approximately 1 in 5.5 HSC lack CD90 expression.[9] Those studies used single cell isolation using flow cytometry sorting to identify the engraftment capability of each cell subset. However, this study utilized a CD34+ CD38- CD90+ definition of an HSC when assessing HSC viability before and after storage.

In addition to progenitor cells, the transplanted graft contains a mixture of mature WBC (CD45+) that are not responsible for engraftment, but are important in other ways. The mature population includes T-cells, which are important for some clinical practices as donor lymphocyte infusions (DLI) to combat graft rejection post-transplant. Thus, it is important to know whether progenitor cells and mature cells survive cryopreservation and storage.

**Cryopreservation history**

Cell storage conditions designed to allow cell survival after storage were studied early in the 18$^{th}$ century, when Reaumur[12] and Spallanzani[13] described the effects of cold temperature storage on cell viability. By 1949, Polge, Smith, and Parkers demonstrated the revival of bull spermatozoa after storage at low temperatures and discovered the first cryoprotective agent (CPA), glycerol.[14] This protocol was applied to human sperm cells, in 1953, by Sherman et al, who established the first sperm bank.[13] Later, in 1955, Barnes and Loutit started the cryopreservation of HSC using glycerol.[15] In 1959, Lovelock and Bishop introduced dimethylsulfoxide (DMSO) as a CPA and compared it with glycerol using the extent of hemolysis of red blood cells as a measure of cell damage caused by freezing.[16]

There are two kinds of cryoprotective agents (CPAs), those that are membrane permeable (e.g. glycerol and DMSO), and those that are non-permeable (e.g. Dextran and Hydroxyethyl starch (HES)). Permeable CPAs are characterized by having a low molecular weight, high solubility in aqueous electrolyte solutions, and the ability to permeate living cells, while non-permeable CPAs have a high molecular weight, which stabilizes the cell membrane of the frozen cells. Non-permeable CPAs augment the effectiveness of the membrane permeable CPAs and allow the use of lower concentrations of membrane permeable CPA. Together, the CPAs help to prevent cell injury caused by the formation of intra- and extra-cellular ice crystals, and also help control the rate of water transport into and out of the cell during freezing, which decreases the toxic effect of hypertonic concentrations of electrolytes and other substances that occurs as cells freeze. The benefit of DMSO over other CPAs is having a greater permeability in living cells. Five to ten percent DMSO is the most commonly used cryoprotective agent. [17]

A slow, gradual addition of DMSO is used to protect cells from damage caused by osmotic stress and the exothermic reaction that occurs as the concentrated DMSO solution mixes with the cell solution.

Lionetti first introduced the use of DMSO and HES in the cryopreservation media for granulocyte cryopreservation in 1975. [18] In 1980 he found that adding human albumin to the cryopreservation medium acts as a cell membrane stabilizer. [19] Stiff and colleagues introduced cryopreservation of bone marrow using DMSO, HES and human albumin in 1987. [20] Additional studies performed in 1991, by Makino and associates to determine the optimal concentrations of DMSO for stem cells cryopreservation media, found that 5% DMSO and 6% HES were optimal. [21]

Cryopreservation protocols for HSC have remained relatively unchanged since the first cord blood-banking program commenced in 1992.[22] New approaches and methods for cryopreservation are needed to: decrease preparation and processing time, improve post-thaw recovery and viability, and reduce DMSO toxicity. Improvements in the materials (or methods) used to cryopreserve HSC/P products that result in better post-thaw viability and recovery may enhance the engraftment of transplanted HSC/P products. In the current practice, cryopreservation media are not standardized between centers. Generally, it is composed of 5 or 10% DMSO, balanced salt solutions (e.g. Normosol-R or Plasma-Lyte A), an anticoagulant (anticoagulant citrate dextrose (ACD-A)) and a protein source such as human plasma or human albumin. Commercially available cryopreservation and hypothermal storage media will help in standardizing the cryopreservation and transportation procedures among stem cell processing centers worldwide.

**Transport media**

The availability of international HSC donation programs increases the demand for better quality transport media. The National Marrow Donor Program (NMDP) outcome data showed that more than 6,200 allogeneic transplants occurred in 2011 and more than 6,700 in 2012. Also, demand for allogeneic transplants is increasing by nine percent annually.[8] Cells are exposed to numerous physical and chemical stresses during transport that can result in changes in membrane-mediated ion transport and intracellular acidosis (pH=4)[23-24] and ultimately, loss of viability. Transport media are used to protect the cells from these stresses as it maintains the ion balance, works as a free radical scavenger, provides osmotic support and nutrients, and controls stress pathways that are activated by hypothermal storage. Improving the transport media could result in decreased cell injury caused by delay

in transporting an allogeneic graft. It could also ease cellular therapy laboratory operations, as it would be possible to delay product processing and allow for overnight storage and pooling of two consecutive peripheral blood stem cell products. Previous studies performed to determine the stability of HSC products in transport media during overnight storage at 4°C prior to cryopreservation have shown that overnight storage has no deleterious impact on short- or long-term engraftment [25], however improving the quality of the transport media might allow products to remain viable for an even longer period of time in instances of transport delays and might someday allow transfusion of fresh autologous products. This would reduce patient exposure to cryoprotectants, which have an associated toxicity risk, and decrease serious adverse reactions after infusion of cryopreserved products. [26]

Previous studies comparing different transport media showed that hypothermic storage using HypoThermosol-FRS® (HTS-FRS®) for 2 and 4 days results in approximately 100 % and 85 % stromal cell recovery, respectively. [27]

Two additional studies have shown that the use of HTS-FRS® results in improved viability and metabolic activity of the transported and stored cells and tissues when compared with other media. [28-29]

## HYPOTHESIS

Commercially available storage media (CryoStor® and HTS-FRS®) designed specifically for cryopreservation and hypothermal storage will improve the viability and functionality of HSC/P subsets compared to conventional cryopreservation and hypothermal storage media.

**Specific aims**

**Aim1: To determine the effect of different cryopreservation media on post thaw viability, recovery and functionality of recovered cell subsets**.

For this aim, post thaw samples were obtained from cord blood units (CBU) that were cryopreserved using CS5®, CS10® or conventional media. Samples were analyzed for viability, recovery and functionality of hematopoietic stem and progenitor cell subsets. In-vitro progenitor cell function assays were utilized to test the cells' ability to form colonies and to proliferate in culture. A colony-forming unit (CFU) assay was used to determine proliferative potential of committed hematopoietic progenitor cells, whereas the cells' ability to proliferate in media in response to a cytokine combination (stem cell factor (SCF), Flt3-ligand and thrombopoietin (TPO)) intended to induce the proliferation of HSCs and immature progenitors, but not committed progenitors, was used to measure stem cell function.

**Aim2: To determine the effect of using different transport media on protecting viable cells during transportation and hypothermic storage of HSC products.**

For this aim, CBU were processed and stored at 4-6°C in either HTS-FRS® or conventional Plasma-Lyte A/0.5 % HSA medium. Samples were tested after refrigerated storage for 24, 48 and 72 h for viability, recovery of viable cells, and functionality in the same manner as for aim 1 above.

## RESEARCH PLAN/OBJECTIVES

The project had two objectives:

1. , Analysis of performance of different cryopreservation media for UCB HSC/P.

2. Analysis of CBU stored at a refrigerated temperature in different transport solutions.

For each of them, the recovery and viability of total nucleated cells (TNC), viability and recovery of mature and immature cell subsets, clonogenic capacity and proliferation potential were measured after storage to determine whether using a commercially available cryopreservation (CryoStor®) and transport (HypoThermosol®) media improves post storage viability, recovery and functionality.

The research plan is outlined in Figure 2.

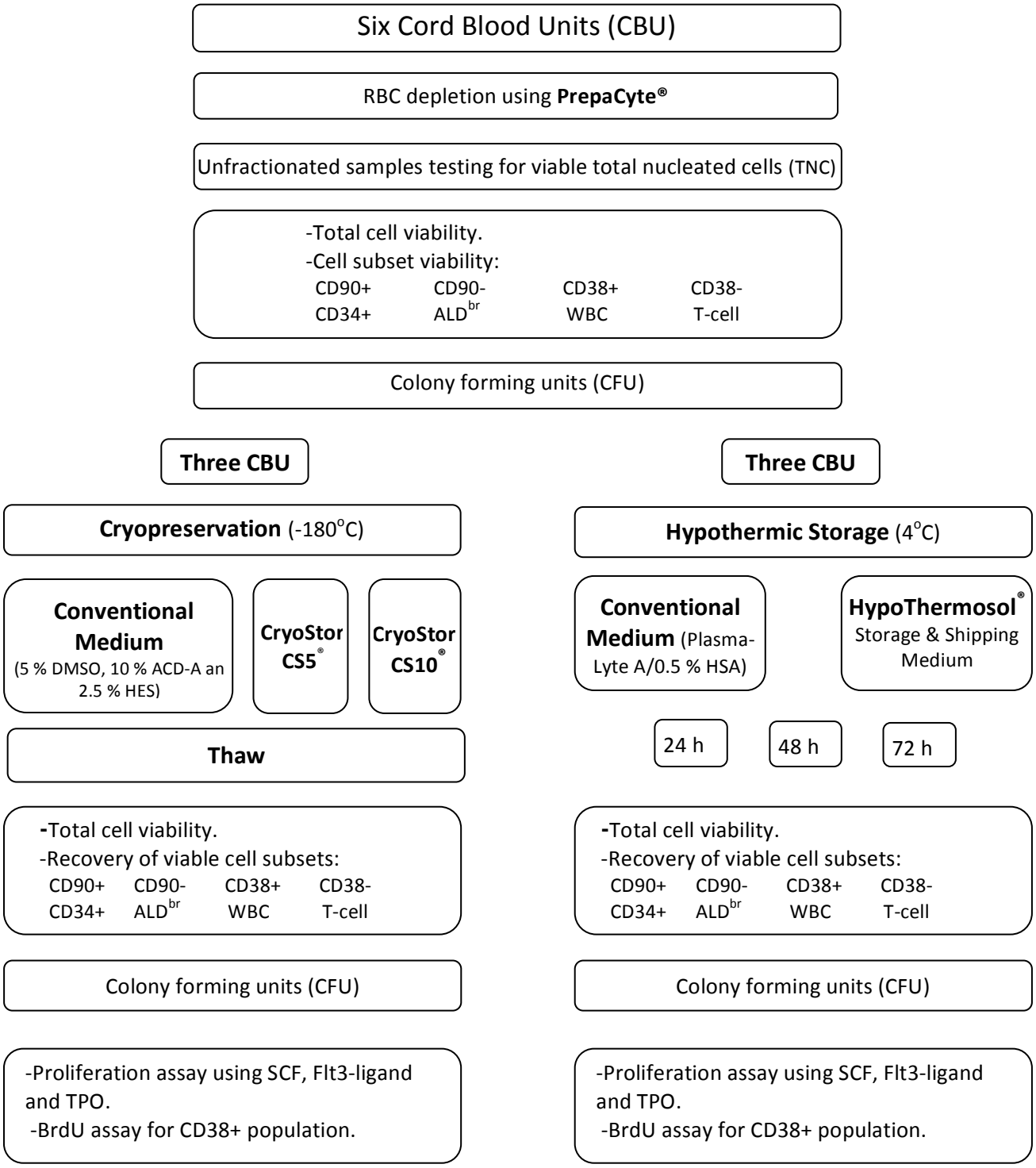



**Figure 2: Illustration of the research plan.**

## MATERIALS

### Umbilical cord blood units (CBU)

Six CBU were used as a representative source of HSC for analysis. The CBU were obtained from deliveries at Christ Hospital through Cincinnati Children's Hospital-Translational Core Laboratories Cell Processing Core through an IRB-approved volunteer donation protocol that allows investigators to request and receive CBU anonymously (CCHMC IRB approval # 00002988). Units were eligible for the study if they were collected within 24 h before processing; CBU had TNC count ≥ 300 x $10^6$ with at least 90 % viability.

### PrepaCyte®

A commercially available kit containing PrepaCyte® (PrepaCyte® BioE, St. Paul, MN) was used to RBC reduce CBU prior to storage. PrepaCyte® is a non-density based buffer that enables rapid and efficient removal of red blood cells (RBC) by aggregating and sedimenting RBC. It allows the recovery of the white blood cells (WBC)(Figure 3). [30]

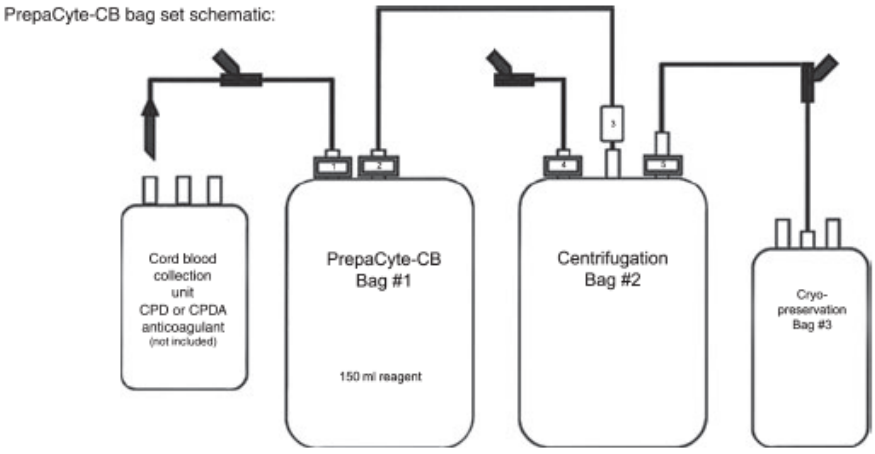



**Figure 3: PrepaCyte® CB bag set schematic.**

**Conventional cryopreservation medium**

The conventional cryopreservation medium is composed of 5 % DMSO (Stemsol™, Protide Pharmaceutical Inc., Lake Zurich, IL), 2.5 % hydroxyethyl starch (HES, 6 % hetastarch in 0.9 % sodium chloride, Hospira, Lake Forest, IL), 10 % ACD-A (acid citrate dextrose, solution A, CaridianBCT, Lakewood, CO), 10 % human serum albumin (HSA), (Catalog # 2G0201 25 % solution, Baxter Healthcare Corporation, Deerfield, IL) and Plasma-Lyte A (Baxter Healthcare Corporation, Deerfield, IL).

Plasma-Lyte A is an electrolyte solution similar in composition to the extracellular fluid. It contains sodium, potassium, magnesium, chloride, acetate, sodium chloride, sodium gluconate, sodium acetate trihydrate, potassium chloride, magnesium chloride and gluconate. It has a pH of 7.4 and an osmolality of 294 mOsm/L. ACD-A consists of dextrose monohydrate, sodium citrate dihydrate and citric acid anhydrous in water for Injection.

**CryoStor®**

CryoStor is a commercially available, animal protein free, and serum free cryopreservation medium (BioLife Solutions) designed to prepare and preserve cells in ultra-low temperature environments (-80°C to -196°C); CryoStor is composed of dextran-40, sodium, potassium, calcium, magnesium, phosphate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), lactobionate, sucrose, mannitol, glucose, adenosine and glutathione. It has a pH of 7.6 and an osmolality of 360 mOsm/kg $H_2O$. [31] It was engineered to maintain a cell's osmotic balance under low temperature conditions, thereby reducing osmotic gradients, which lead to the onset of cell death via apoptosis, necrosis, and secondary necrosis. [17] CryoStor® is pre-formulated with different concentrations of DMSO. In this study, CryoStor media with 5 and 10 % DMSO (CS5 and CS10) were used.

**Thawing medium**

A thawing media (TM) consisting of 30 % Plasma-Lyte A, 6 % dextran, 2.5 % HSA and 10 U/mL of heparin (Hospira) was prepared and chilled at 4-6°C for at least 20 min prior to use.

**Conventional hypothermal-storage medium**

A conventional hypothermal storage (transport) medium was used, consisting of Plasma-Lyte and 0.5 % HSA (25 % solution) (both Baxter Healthcare).

**HypoThermosol®**

HypoThermosol-FRS® (HTR-FRS®, BioLife Solutions) is a commercially available transport medium that is composed of dextran-40, sodium chloride, potassium chloride, calcium chloride, magnesium chloride, dihydrogen phosphate, bicarbonate, HEPES, lactobionate, sucrose, mannitol, glucose, adenosine and glutathione. It has a pH of 7.6 and an osmolality of 360 mOsm/kg $H_2O$. [29]

## METHODS

All procedures that involve an open system were performed aseptically under a class II biological safety cabinet (BSC, SteriGard Hood type A/B3, Baker Co. Inc., Sanford, Maine).

## Cord blood processing

Upon receipt of a CBU, a 0.4 mL sample was collected through a blood bag spike adaptor (catalog # 1C8687, Baxter Healthcare) to determine the TNC count (automated cell counter, COULTER AC.T 5 diff CP, BECKMAN COULTER, Brea, CA) and the viability (flow cytometry, FACSCanto II, BD Biosciences, San Jose, CA).

RBC depletion was performed using a commercially available kit (contains PrepaCyte® buffer). The manufacturer's recommended protocol was followed.

If the minimum criteria were met (cell count and viable TNC percentage), the CBU was transferred to processing bag number 1 that contained the PrepaCyte solution using an attached spike. For optimal recovery, a portion of the reagent–cord blood mixture—was drained back into the collection bag, mixed and transferred back into bag number 1. The primary collection bag was then rinsed with 5 mL of Plasma-Lyte A solution. The tubing between the primary collection bag and processing bag number 1 was heat-sealed and the primary collection bag was discarded. The reagent-cord blood mixture was mixed manually for 15-20 min. After mixing, the bag set was suspended on a manual plasma expressor (Fenwal Laboratories, Deerfield, IL) for 45-60 min. The TNC rich supernatant was then transferred slowly to bag number 2 using the plasma expressor and a hemostat to control the flow rate. The second bag along with its contents was centrifuged at 400 x g for 10 min. (Beckman GS-6R centrifuge, BECKMAN COULTER Inc.) with the brake off to avoid disruption of the pellet. Once more using the plasma expressor, the second unwanted supernatant was transferred back to bag number 1. The final product volume was measured by transferring it aseptically through the kit port to a 30 mL syringe (Catalog # 309650, BD, Franklin Lakes, NJ). The RBC-depleted product was then returned to bag number 2.

After the CBU had been processed, samples were taken for cell counts, flow cytometry (subsets viability and recovery analyses), clonogenic capacity, and proliferation potential before cryopreserving (freezing) or hypothermal storage at $4^{o}$C.

## Cryopreservation

Three of the RBC-depleted CBU were cryopreserved in cryovials. Six 5 mL cryovials (Catalog # CLS430663, Corning Inc., Corning, NY) were prepared; labeled A1, A2, B1, B2, C1 and C2. Each set of two was cryopreserved using different media. Vials A1 and A2 were processed with the conventional media, B1 and B2 with CS5 and C1 and C2 with CS10. Equal volumes of the final product were transferred to each vial. The cryopreservation volume was reduced by centrifuging the vials for 10 min. at 200 x g. The supernatant was then aspirated using a 5 mL graduated pipette (Corning Inc.) and each vial processed with the corresponding cryopreservation media to a final concentration of 5-50 X $10^{6}$ NC/mL per 3 mL cryopreserved product.

After adding the cryopreservation media, vials were stored overnight in a -70°C freezer in a freezing container that provides a repeatable -1°C/min cooling rate (Mr. Frosty, Thermo Fisher Nalgene, Waltham, MA) before transferring to liquid nitrogen storage (vapor phase). After at least 24 hrs in liquid nitrogen, one set of the cryopreserved tubes (A1, B1 and C1) was thawed, washed, and diluted prior to testing, and the second set of tubes was left as a backup.

For the conventional cryopreservation process, cryopreservation medium containing DMSO and HES (300µL and 900µL respectively) was prepared and pre-chilled at 4-6°C for at least 15 min. before adding it to the cell suspension. Cell pellets were re-suspended with 500 µL

Plasma-Lyte, 300 µL 25 % HSA, 800 µL 6 % HES and 300 µL ACD-A and then chilled for 10 min. at 4-6°C. After chilling, 600 µL of the pre-cooled cryopreservation media were slowly added to the cell suspension. The cryovials were then placed in a 5 mL Mr. Frosty container and immediately placed in a -70°C freezer.

For B1 and C1, vials designated for CS5 and CS10, cell pellets were re-suspended with 2.5 mL of pre-chilled CS5® or CS10® (at 4-6°C) to reach a final volume of 3 mL. The vials were then placed in a 5 mL Mr. Frosty and further chilled for 10 min. at 4-6°C. then placed in a -70°C freezer.

**Thawing procedure**

The cryopreserved vials were thawed after a minimum of 24 h in vapor phase liquid nitrogen storage. Immediately after thawing, cryovials were diluted 1:12 with cold TM in order to avoid drastic changes in osmolality and prevent toxicity from the DMSO.

The cryovials were always handled in parallel and the thawing procedure was standardized for all of the cryopreserved samples. The samples were removed from a liquid nitrogen freezer, then thawed in a 37°C water bath with gentle swirling, until all visible ice was melted. Vials were then immediately placed under a BSC to be diluted with the precooled TM. One mL of the TM was added drop wise to minimize osmotic shock to the cryovials, and then its entire content transferred to a 50 mL labeled sterile centrifuge tube (catalog # C1062-P, Denville Scientific Inc., South Plainfield, NJ). The cryovials were rinsed with 1 mL of the TM and 31 mL of the TM was added slowly (drop wise) to the 50 mL centrifuge tube. Immediately after the addition of TM, 0.4 mL post-thaw samples were taken for automated cell count and flow cytometry testing. The diluted post-thaw samples were then incubated for 30 min. at RT. These were then centrifuged at 200 x g for 10 min. The supernatant was

discarded and the cell pellet re-suspended with 5 mL of the TM. The TNC count was calculated and samples for CFU and proliferation assay were taken.

## Hypothermal storage.

Three CBU were processed for hypothermal storage. After calculating the TNC, the post-processing product volume was divided equally into two 50 mL centrifuge tubes A and B. The tubes were centrifuged at 300 x g for 10 min. to reduce the volume. The volume of hypothermal storage media was calculated to obtain a final concentration of 50 X $10^6$ NC/mL. Each cell pellet was re-suspended with the corresponding storage media and mixed well. The tube content was transferred into two 150 mL transfer bags (catalog # 4R2011, Baxter). Bags were then stored in a monitored 4-6°C refrigerator. Samples were obtained periodically after 24, 48 and 72 h in order to evaluate different storage conditions.

## Flow cytometry

Flow cytometry utilizes monoclonal antibodies conjugated with fluorochromes, which bind to the targeted antigens and emit florescence at different wavelengths when passed through a laser beam. A flow cytometer was used for cell subset identification, viability measurement, ALDH quantitation (ALDEFLUOR assay), and BrdU incorporation.

To evaluate the various cell subsets, pre and post storage samples were stained with fluorochrome-conjugated antibodies against different surface antigens (anti-CD45-PE (Phycoerythrin), anti-CD34-APC-Cy7 (Allophycocyanin with cyanin-7), anti-CD38-APC (Allophycocyanin), anti-CD90-PE, anti-CD3-APC (Table 1) in different combinations. Viability was measured using 7-AAD (BD Pharmingen, San Jose, CA) and annexin-V-FITC (Fluorescein

isothiocyanate). The ALDEFLUOR assay (STEMCELL Technologies, Vancouver, BC, Canada) was utilized to measure ALDH content.

**Table 1: List of antibodies against HSC/P subsets surface antigens used for flow cytometry.**

| Product Name | Fluorochrome | Clone | Producer |
|---|---|---|---|
| **CD45** | PE | TU116 | BD Pharmingen™ |
| **CD34** | APC-Cy7 | 581 | Biolegend |
| **CD38** | APC | HB7 | BD Pharmingen™ |
| **CD90** | PE | 5E10 | e Bioscience |
| **CD3** | APC | UCHT1 | BD Pharmingen™ |
| **Annexin-V** | FITC | VAA-33 | BD Pharmingen™ |

For each sample, a cell suspension containing 20 x $10^6$ NC/mL in Dulbecco's phosphate buffered saline (D-PBS) and 2 % fetal bovine serum (FBS) (both Sigma Life Science, St. Louis, MO) was prepared. Eight testing tubes with different cell concentrations were prepared. Six tubes were used for HSC/P subset identification, one for CD3 evaluation, and one for the ALDEFLUOR assay. One hundred microliters of the prepared suspension were transferred to the first set of tubes (1-3) to obtain 2 X $10^6$ cells for staining. Two hundred microliters were transferred to the second set of tubes (4-6) to obtain 4 X $10^6$ cells. Fifty microliters were transferred to the CD3 evaluation tube to obtain 1 X $10^6$ cells concentration, and 150 µL were transferred to the ALDEFLUOR testing tube to obtain 3 X $10^6$ cells for staining.

**Staining procedure**

Cells were suspended in 3 mL of D-PBS supplemented with 2 % FBS and centrifuged at 220 x g for 7 min. The supernatants were then discarded and the cell pellets were re-suspended directly with the isotype control or murine anti-human monoclonal antibodies (20µL per $10^6$ cells) and incubated for 20 min. in a 4-6°C refrigerator. The list of antibodies added to each tube is shown in Table 2. Cells were then incubated for 15 min. at RT with lysing buffer (BD Bioscience, San Jose, CA) to lyse the residual RBC. Tubes were then centrifuged at 220 x g

for 7 min. and the cell pellets washed once with 1 mL of D-PBS/2 % FBS solution. After washing, the cell pellets were re-suspended in 100 µL 1:10 annexin-V binding buffer (BD bioscience, San Jose, CA). To standardize the volume of viability staining added to each tube, 7-AAD and annexin-V solution was prepared in a staining buffer (SB) (BD Pharmingen™, San Jose, CA) and proper amount of the solution was added to each tube. The cell suspensions were then incubated for 15 min. at RT and then placed on ice and analyzed immediately. Data acquisition was performed using a dual laser FACSCanto II (BD bioscience) flow cytometer and data analyzed using FACSDiva software (BD bioscience, San Jose, CA).

**Table 2: Flow cytometry tubes set-up.**

| Tube# | CD45-PE | CD3-APC | CD34-APC-Cy7 | CD38-APC | CD90-PE | Iso- APC-Cy7 | Iso-APC | Iso-PE | AnnexinV-FITC+7-AAD | ALDEFLUOR |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | X | | | | | X | X | | X | |
| **2** | X | | X | | | | X | | X | |
| **3** | X | | X | X | | | | | X | |
| **4** | | | X | X | | | | X | X | |
| **5** | | | X | X | X | | | | X | |
| **6** | | | X | X | X | | | | X | |
| **CD3** | | X | | | | | | | X | |
| **ALDEFLUOR** | X | | X | X | | | | | | X |

**Aldehyde dehydrogenase enzyme**

A high level of aldehyde dehydrogenase (ALDH) activity has been proposed to be a common feature of stem cells.[11] The ALDEFLUOR™ reagent kit (STEMCELL Technologies) was used in combination with anti-CD45-PE, anti-CD34-APC-Cy7 and anti-CD38-APC to detect cells with high ALDH levels. The activated ALDEFLUOR™ Reagent, BODIPY™- aminoacetaldehyde (BAAA) is a fluorescent non-toxic substrate for ALDH, which freely diffuses into intact and viable cells. In the presence of ALDH, BAAA is converted into BODIPY™-aminoacetate (BAA), which is retained inside the cells. The amount of fluorescent reaction product is proportional to the ALDH activity in the cells and is measured using a flow cytometer.

To stain the cells with ALDEFLUOR, 3 X $10^6$ cells were washed with 3 mL of D-PBS/2 % FBS. RBCs were lysed using 2 mL lysing buffer and washed with 1 mL of the D-PBS /2 % FBS. Cells were then diluted with 1 mL of pre-warmed – at RT— ALDEFLUOR™ buffer and 5 µL of activated ALDEFLUOR™ reagent. Five hundred microliters of the suspension were immediately transferred to 1.5 mL microtiter tube that contained 5 µL of ALDEFLUOR™ control reagent. The rest of the suspension was transferred to another 1.5 mL microtiter tube and both incubated for 30 min. at 37°C. After incubation, tubes were centrifuged for 7 min. at 220g then cells were stained with CD45-PE, CD34-APC-Cy7 and CD38-APC (Table 2).

After staining cells were washed in 1 mL of the ALDEFLUOR™ buffer then re-suspended with 100µL of the ALDEFLUOR™ buffer and stained with 10µL 7-AAD for 15 min. After incubation 400 µL of the ALDEFLUOR™ buffer was added and the tubes kept on ice until acquisition.

**Gating strategies**

**Cell subset identification**

Flow cytometry was used to identify hematopoietic cell subsets that vary in maturity. The CD34+, CD38-and CD90+ cell is the most immature cell while the CD3+ T-cell is the most mature cell of interest. The first set of tubes (1-3) was used to identify the WBC (CD45+), and the CD34+ (CD45+ and CD34+), CD38- (CD45+, CD34+ and CD38-) and CD38+ (CD45+, CD34+ and CD38+) progenitor cells. The second set of tubes (4-6) was used to identify the more primitive CD90- (CD34+, CD38- and CD90-) MPP and the CD90+ (CD34+, CD38- and CD90+) stem cells. The T-cells (CD3+) were identified from the CD3 tube. The cell subset phenotypes are summarized in Table 3.

**Table 3: Cell subset phenotypes.**

| Cell subset | CD Markers |
|---|---|
| **CD90+** | CD34+, CD38-, CD90+ |
| **CD90-** | CD34+, CD38-, CD90- |
| **ALD$^{br}$** | CD45+, CD34+, CD38-, ALD$^{br}$, 7-AAD- |
| **CD38-** | CD45+, CD34+, CD38- |
| **CD38+** | CD45+, CD34+, CD38+ |
| **CD34+** | CD45+, CD34+ |
| **WBC** | CD45+ |
| **T cell** | CD3+ |

Different gating strategies were followed for the different tube sets. The International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines for CD34+ determination were followed for the first set of tubes (1-3) (Figure 4-A). [32] For the next set of tubes (4-6), the CD34 gate was used directly after the light scatter gate, followed by CD38 and CD90 gates (Figure 4-B). The same strategy used for the first set of tubes (1-3) was followed for The ALDEFLUOR™ assay analysis. The ALD$^{br}$ region was determined from the control tube. The ALD$^{br}$, 7-AAD- cells were identified from the CD45+, CD34+ and CD38- population (Figure 5).

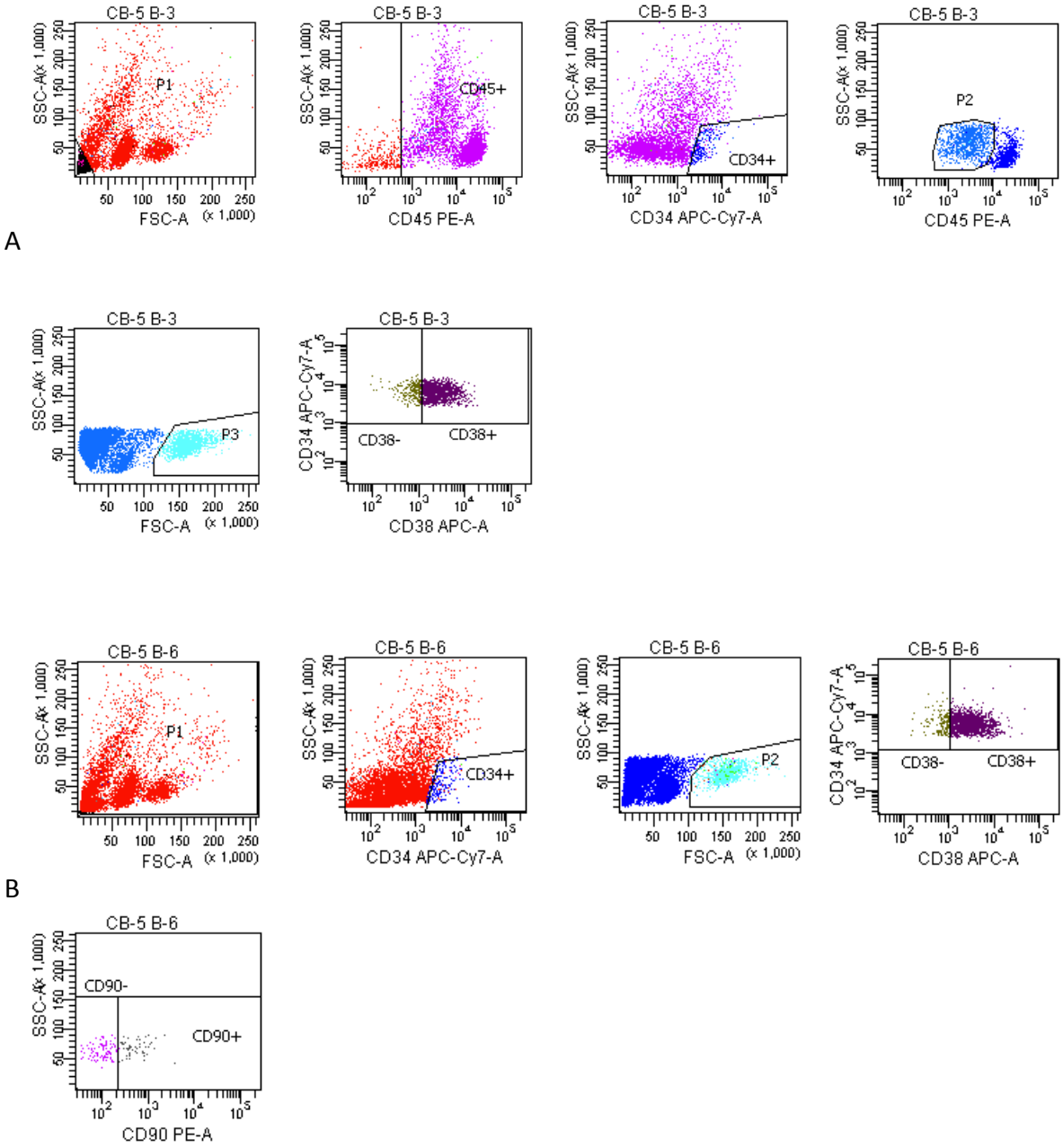
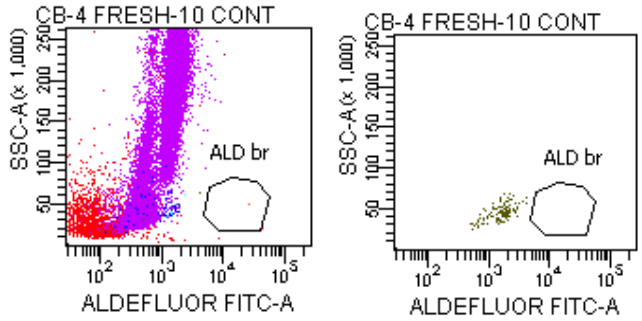



**Figure 4: Gating strategies**

(A) Guidelines followed for the first set of tubes (1-3) P1 used to exclude RBCs and debris followed by CD45 then CD34. P2 used to exclude the CD34+, CD45 bright cells and P3 in light scatter plot used to exclude debris. The CD38 fluorscence was gated on the CD45+ CD34+ (P3) population. .(B) Gating strategies for the second set of tubes (4-6). P1 used to exclude RBCs and debris followed by CD34 then P2 to exclude debris. Then CD38 gates were determined from P2 population, and the CD90 fluorescence was gated on the CD38- population. FSC-A = forward scatter (area). SSC-A = side scatter (area).

A




B

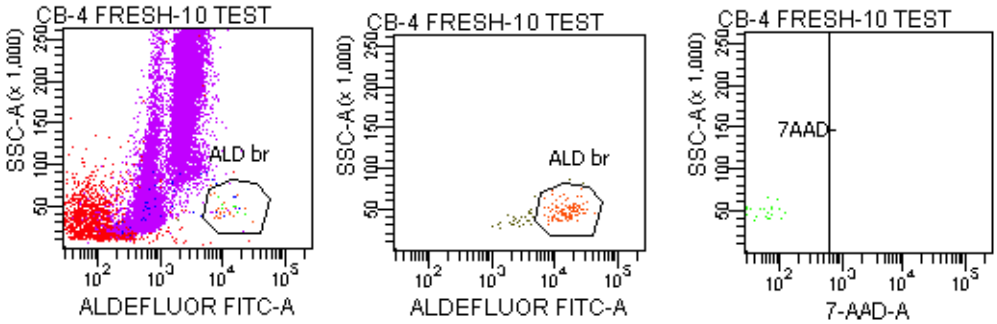



**Figure 5: ALDEFLUOR assay evaluation**

Measurement of ALDH content of CD34+ CD38- cells. (A) ALDEFLUOR-FITC vs side scatter plot used to determine the ALD$^{br}$ region from the control tube. (B) The ALD$^{br}$ cells are determined from the testing tube.

The CD3+ population was evaluated from tube stained with anti-CD3-APC, 7-AAD and annexin-V-FITC (Figure 6).

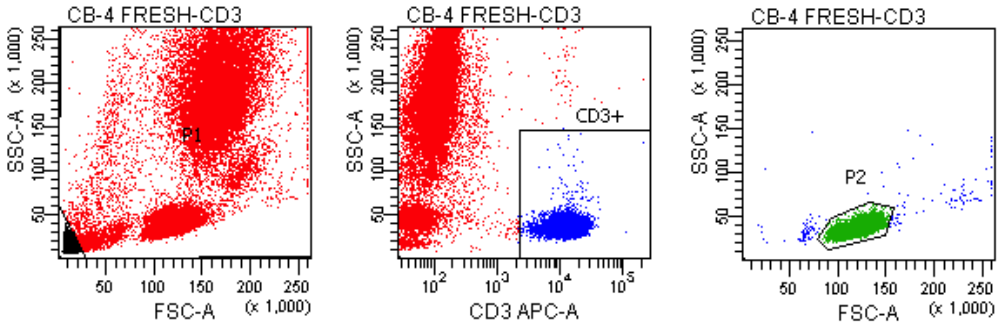



**Figure 6: Gating strategy for the CD3+ population.**

P1 was used to exclude part of the RBC. CD3+ population was selected and gated back to a light scatter plot to narrow it to the lymphocyte region. FSC-A = forward scatter (area). SC = side scatter.

**Viability evaluation**

In this study, viable cells were defined as 7-AAD negative and annexin-V negative. 7-AAD is measuring the cells' viability by testing their ability to uptake the viable stain 7-AAD. Since viable cells have an intact membrane, they will not be stained with 7-AAD. Annexin-V is measuring an Apoptosis (Apo) marker, which is loss of membrane phospholipid symmetry. Phosphatidylserine (PS) is normally expressed in the cytoplasmic layer of the plasma membrane. Upon apoptosis, PS is trans-located to the outer layer. PS expression plays an important role in the recognition and removal of apoptotic cells by macrophages.[33] Annexin V dependent phospholipid-binding protein has a high affinity for PS, which makes it possible to measure apoptosis. Using annexin-V in combination with 7-AAD allows us to identify the apoptotic cells, which are the 7-AAD negative and annexin-V positive population (Figure 7).[34] The percentage of different cell subsets at apoptotic and necrotic stages was compared among different storage conditions.

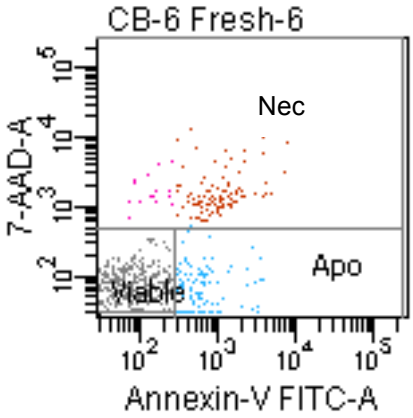



**Figure 7: Evaluation of viability.**
7-AAD and annexin-V FITC plot used to identify the viable, apoptotic (Apo) and necrotic (Nec) populations.

**Recovery calculations**

Post storage total nucleated cell (TNC) recovery was calculated using the following formula:

$$\% \text{ TNC recovery} = \frac{\text{Post storage TNC count}}{\text{Fresh sample TNC count}} \times 100$$

Recovery of various cell subsets was calculated using the following equation:

$$\% \text{ Recovery of viable cells subset \%} = \frac{\text{Post storage cell subset \% X Subset viability \% X Post storage TNC count}}{\text{Fresh sample cell subset \% X Subset viability \% X Fresh sample TNC count}} \times 100$$

Analysis of viable cell recovery of the various cell subsets was performed to determine whether the cryopreservation or storage conditions tested have more of an effect on one population or another, since total cell viability might be high, but progenitor cell viability might not.

## In-vitro functional assessment

**Colony forming unit assay**

A CFU assay was performed for post-processing samples, all post-thaw samples and for 48 h and 72 h hypothermic stored samples. The CFU assay was performed using methylcellulose colony assay medium (Methocult® H4434, Stem Cell Technologies, Vancouver, Canada), which is supplemented with SCF, EPO, IL-3 (interleukin 3) and GM-CSF (Granulocyte-macrophage colony stimulating factor). Two cell concentrations ($1X10^4$ cells/mL and $3X10^4$ cells/mL, in IMDM) were tested to ensure a sufficient number of colonies to count. The cells were inoculated into 3 mL Methocult® H4434 medium. Blunt end needles (Stem Cell Technologies) attached to 3 mL syringes were used to transfer 1.1 mL to 35 mm culture dishes [35] (Stem Cell Technologies). Culture dishes were left in a humidified 37°C incubator

with 5 % $CO_2$ (OLYMPYS IMT-2) for 14 days. Burst-forming unit (BFU), colony-forming-unit granulocyte monocyte (CFU-GM), and colony-forming-unit granulocyte erythroid monocyte megakaryocyte (CFU-GEMM) colonies greater than 50 cells were counted using an inverted microscope. The average colony count from 2 plates was calculated and used for analysis.

**Proliferation assay**

Twenty two million nucleated cells were cultured in six well plates containing 2 mL/well of StemSpan® SFEM serum free medium (STEMCELL Technologies) with 30 µg/mL Human LDL (low density-lipoprotein) (STEMCELL Technologies) and a cytokine cocktail (StemSpan® CC110) that included stem cell factor (SCF), Flt3-ligand and thrombopoietin (TPO) [36], at a 1:100 dilution—according to the manufacturer's instructions. Plates were incubated at 37°C in 5 % $CO_2$ and cells were analyzed after 48 h Bromodeoxyuridine (BrdU) uptake assay (BD Bioscience) accompanied with CD45, CD34 and CD38 staining was used to evaluate the proliferation potential of the CD38+ (CD45+, CD34+ and CD38+) population.

**BrdU incorporation assay**

Bromodeoxyuridine (BrdU) is an analog of the DNA precursor thymidine that is incorporated into newly synthesized DNA as cells progress through the S phase of the cell cycle. The incorporated BrdU is stained with specific FITC conjugated anti-BrdU antibodies. The immunofluorescent staining of incorporated BrdU and flow cytometric analysis provided a high-resolution technique to determine the frequency cells that have synthesized DNA during the S phase of the cell cycle (Figure 8).

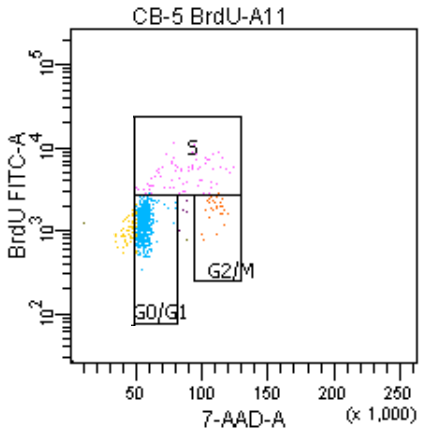



**Figure 8: Flow cytometry analysis of BrdU incorporation assay.**
BrdU-FITC and 7-AAD plot was used to determine the cell cycle stages. G0/G1 is the cell's quiescence stage (non-proliferating cells). S phase is the DNA synthesis phase and G2/M is after DNA proliferation when the cell's DNA duplicated.

To perform the BrdU uptake assay in proliferating cells, the cultured cell suspension was transferred to 5 mL sterile tubes (Becton Dickinson, Franklin Lakes, NJ) centrifuged for 5 min. at 170 x g then washed once with 2 mL of D-PBS/ 2 % FBS (BD, San Diego, CA). Cells were re-suspended in 1 mL of StemSpan SFEM supplemented with 10 µL of 23X BrdU (1mM in 1X D-PBS) and incubated for 45 min. at 37°C and 5% $CO_2$. Cells were centrifuged at 200 x g for 5 min. then washed with 1 mL of SB then stained with anti-CD45-PE, anti-CD34-APC-Cy7 and anti-CD38-APC. Cells were then suspended with 100 µL of BD Cytofix/Cytoperm™ buffer and incubated for 20 min. at RT for fixation and permeabilization then washed with 1 mL of 1X BD perm/ wash™ buffer. Cells were incubated with 100 µL of BD Cytoperm™ buffer for 10 min. on ice, washed with 1 mL of 1X BD perm/ wash™ buffer and treated with 100 µL of diluted DNase (30 µg) to expose the incorporated BrdU, then stained with 1:50 diluted anti-BrdU FITC conjugated antibodies. To stain the total DNA level, treated cells were stained with 20 µL 7-AAD. Samples were analyzed by flow cytometry to detect the levels of synthesized DNA in the CD45+, CD34+,CD38+ population.

## STATISTICAL ANALYSIS

Data were recorded as mean ± standard deviation (SD). Statistical significance was evaluated using the paired or non-paired Student's t-test and correlation coefficient (R). The significance threshold was set at p-value < 0.05. Data were analyzed using Excel 2011(Microsoft® Excel® for Mac 2011, v14.32 (130206). Microsoft Co. Seattle, WA).

## RESULTS

## RBC depletion

Commercially available PrepaCyte® solution was used to perform RBC depletion. TNC count was performed before and after the process. In fresh CBU, the volume of the unit varied between 89 and 117 mL , and the cell count varied between $340X10^6$ – $820X10^6$ TNC. PrepaCyte® depleted an average of 98% of the RBC, with an average viable TNC recovery of 75 ± 19% (Table 4).

**Table 4: TNC and RBC recovery after RBC depletion.**

| | | Pre-RBC Depletion | Post-RBC Depletion |
|---|---|---|---|
| **Volume (mL)** | | 102 ± 10 | |
| **RBC volume (mL)** | | 18 ± 6 | 0.4 ± 0.1 |
| **RBC recovery** | % | | 2 ± 1 |
| **Viable TNC** | No. | $450X10^6$ ± $160X10^6$ | $320X10^6$ ± $99X10^6$ |
| **Viable TNC recovery** | % | | 75 ± 19 |

CBU processed with PrepaCyte® for RBC depletion. Data expressed as mean ± SD. (N=7).

After RBC depletion, fresh samples were analyzed for the viability of each cell subset. There was a large variation in the various cell subset concentrations within each cord blood unit (Table 5).

**Table 5: Cell subset concentrations in fresh CBU.**

| Cell subset | Cell Surface Antigen | % Concentration |
|---|---|---|
| CD90+ | CD34+, CD38- and CD90+ | 0.013 ± 0.008 |
| CD90- | CD34+, CD38- and CD90- | 0.096 ± 0.048 |
| $ALD^{br}$ | CD45+, CD34+, CD38-, $ALD^{br}$, 7-AAD- | 0.073 ± 0.064 |
| CD38- | CD45+, CD34+, CD38- | 0.090 ± 0.096 |
| CD38+ | CD45, CD34+, CD38+ | 0.175 ± 0.084 |
| CD34+ | CD45+, CD34+ | 0.234 ± 0.171 |
| WBC | CD45+ | 88 ± 7 |
| T cell | CD3+ | 18 ± 10 |

Cell subset concentrations from fresh CBU after RBC depletion. Data expressed as the mean percentage ± SD (N=6).

## Cryopreservation

### Evaluation of viable cell subset recovery

Cryopreserved samples were thawed and diluted 1:12 with pre-cooled TM. Samples were taken immediately after dilution and were analyzed for cell count and flow cytometry.

Total nucleated cell (TNC) recovery was similar in all post-thaw samples. TNC recovery averaged 90% after cryopreservation in the conventional cryopreservation media, 87% when using the CryoStor 5 and 91% when using the CryoStor 10, but these differences were not statistically significant (Table 6). Recovery of viable cells was unexpectedly low with all cryopreservation media. Although there was a trend toward improved post thaw viable cell recovery after cryopreservation in the conventional medium, the differences in the recovery of viable cells between cryostorage media were not statistically significant (p-value 0.2-0.8) (Table 6, Figure 9).

**Table 6: Post-thaw recovery of TNC and viable TNC**

| | | Pre-storage | Post-thaw | | |
|---|---|---|---|---|---|
| | | **Fresh** | **Conventional** | **CryoStor5** | **CryoStor10** |
| **TNC count** | No. | $250X10^6 \pm 38X10^6$ | $55X10^6 \pm 27X10^6$ | $53X10^6 \pm 24X10^6$ | $56X10^6 \pm 20X10^6$ |
| | Recovery % | | 90 ± 7 | 87 ± 6 | 91 ± 6 |
| | P-value | | | 0.8 | 0.5 |
| **Viable TNC** | No. | $197X10^6 \pm 26X10^6$ | $23X10^6 \pm 15\ X10^6$ | $20X10^6 \pm 13X10^6$ | $21X10^6 \pm 10X10^6$ |
| | Recovery % | | 51±5 | 43 ±10 | 48 ± 10 |
| | P-value | | | 0.4 | 0.2 |

TNC and viable TNC count in fresh and post-thaw samples. P-value compared the conventional with CryoStor media. Data are presented as the mean ± SD (N=3).

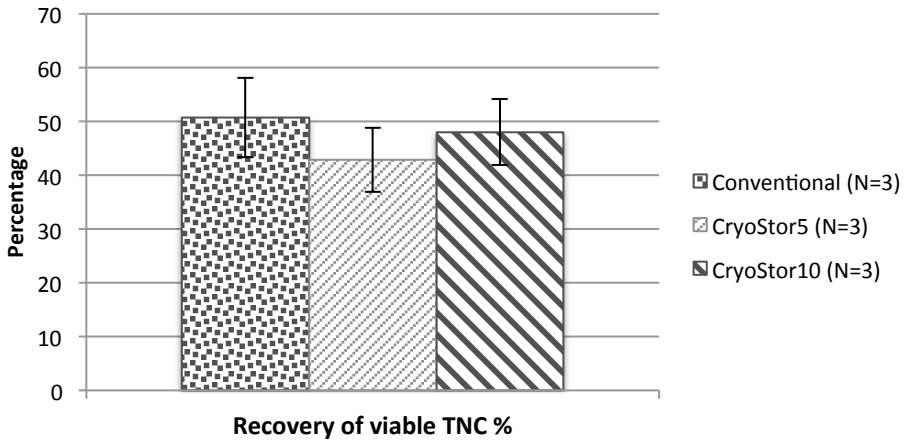



**Figure 9: Post-thaw viable TNC recovery.**
Viable TNC recovery was calculated using the percentage of viable CD45+. Recovery results are expressed as mean ± SD.

The post-thaw viability (%7-AAD neg./annexin-V neg.) of the entire CD45+ WBC population was surprisingly low, ranging from 25 – 50% for the 3 samples, although the difference in post-thaw viability between the 3 samples was not statistically significant. The more primitive hematopoietic progenitor cell subsets (CD90+, CD90-, CD38-, CD38+ and CD34+) had a higher post thaw viability than the CD45+ cells and, although not statistically significant, there seemed to be a trend toward improved post-thaw viability of these subsets when using CryoStor 5. Similarly, the post thaw viability of the mature CD3+ T cell subset was much better than for the entire WBC population and approximately the same as

for the progenitor cell populations, but with this subset, although not statistically significant, it appeared that the post thaw viability was slightly better when using CS 10. The lowest post-thaw viability occurred in the CD90- population, although the number of cells analyzed was quite small and the variability in the measurement quite large, so the difference was not statistically significant. A graphical representation of the post-thaw viability results is shown in Figure 10.

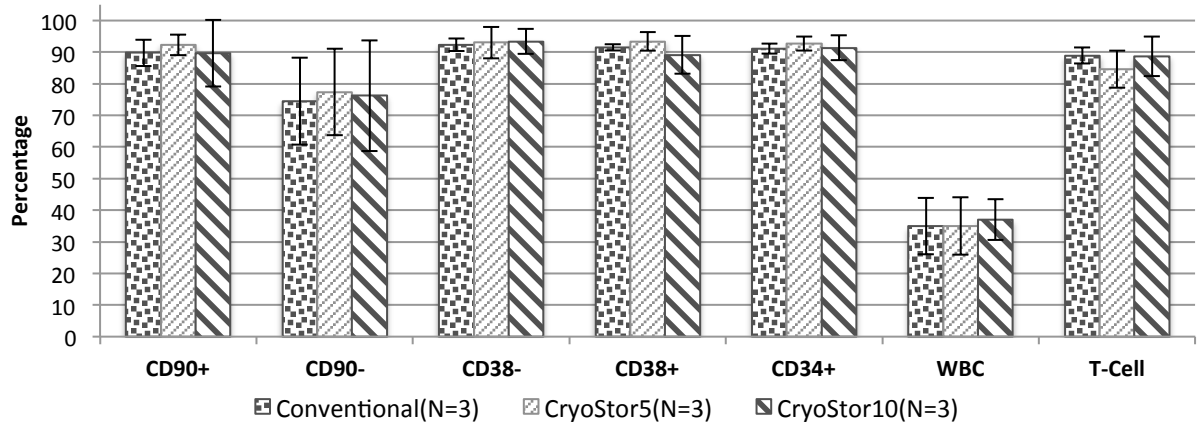



**Figure 10: Viability evaluation of post-thaw samples.**
Viability evaluation using 7-AAD neg./annexin-V neg. Data are presented as percentage mean ± SD.

Since there may be deleterious effects of cryopreservation on cells that go undetected by analyzing post-thaw viability alone, we evaluated the percentage of the cells undergoing apoptosis (%7-AAD neg., annexin-V pos.) after cryopreservation and thawing. In general, there was less apoptosis after freezing and thawing the hematopoietic progenitor cell subsets (CD90+, CD38-, CD38+ and CD34+) , than there was for the CD3+ T cell population or for the entire CD45+ WBC population (Figure 11). As with post-thaw viability, the highest amount of apoptosis amongst the progenitor cell populations occurred with the CD90- population, although again the number of cells analyzed was quite small and the variability in the measurement quite large, so the difference was not statistically significant. There were only small, statistically insignificant differences in the level of apoptosis observed after

freezing with the three different cryopreservation media (p-value 0.3-1). A graphical representation of the percentage of cells undergoing apoptosis after freezing and thawing is shown in Figure 11.

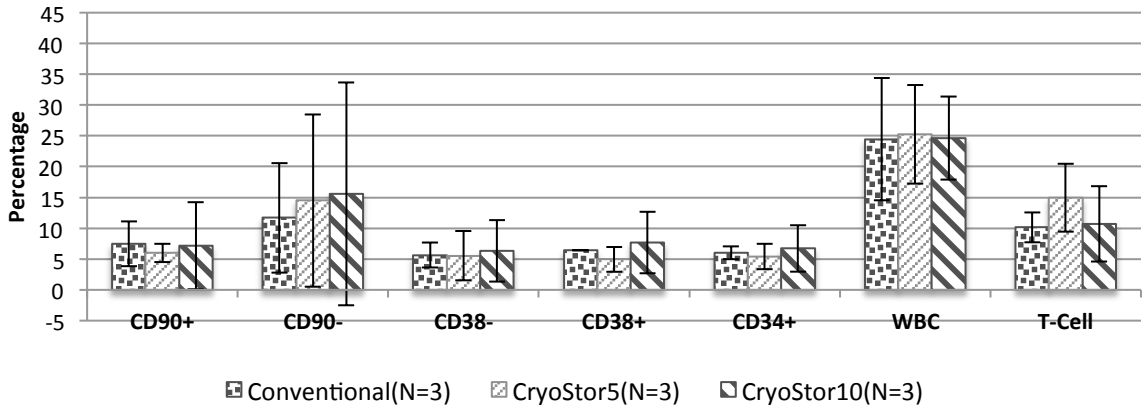



**Figure 11: Evaluation of post-thaw apoptosis.**
Flow cytometric apoptosis evaluation of post-thaw samples. Data are expressed as the mean ± SD.

Recovery of viable cells post thaw varied amongst the various subsets analyzed. Despite a large variability in the results and despite a lack of statistical significance (P-value 0.2-1.0), It appeared that perhaps the CD90+ and CD90- subsets survived the freeze/thaw process better when they were stored in the conventional cryopreservation medium than either of the CryoStor medias, whereas it appeared that the CryoStor 10 media worked better for the CD3+ T cells, and it appeared the other subsets all survived equally in the three cryopreservation medias (Figure 12).

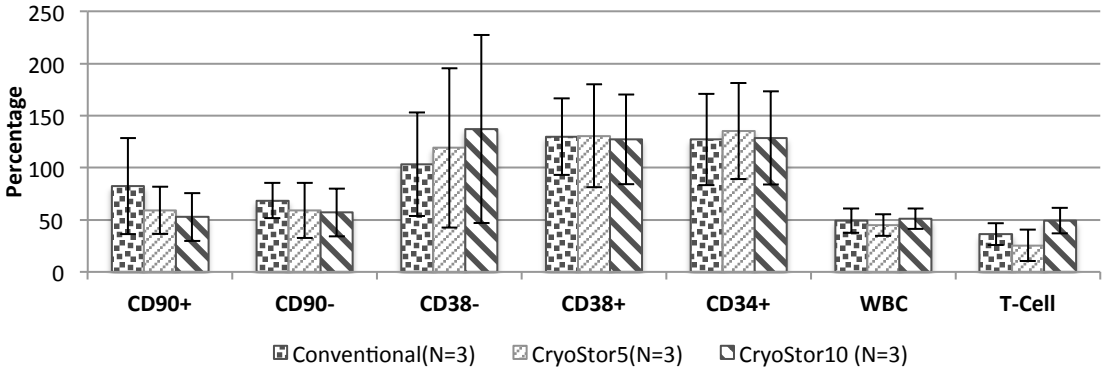



**Figure 12: Post-thaw cell recovery.**
Data are presented as the mean ± SD.

## Recovery of ALDEFLUOR $^{Bright}$ cells

Perhaps because there were so few CD45+, CD34+, CD38- ALD$^{br}$ cells in the 3 cord blood samples tested, there was a large variation in the post-thaw recovery calculations, and therefore no statistically significant differences between the 3 cryopreservation medias tested (Table 7; Figure 13). The post-thaw viability of the ALD$^{br}$ cells was >90% for all three cryopreservation medias also.

**Table 7: Post-thaw ALDEFLUOR evaluation**

| | Fresh | Conventional | CryoStor 5® | CryoStor 10® |
|---|---|---|---|---|
| **ALD$^{br}$ %** | 0.061 ± 0.028 | 0.036 ± 0.021 | 0.029 ± 0.011 | 0.032 ± 0.012 |
| **P-value** | | | 0.7 | 0.8 |
| **Recovery %** | | 74 ± 74 | 59 ± 53 | 65 ± 56 |
| **P-value** | | | 0.8 | 0.9 |

P-values compared the variation in recovery and ALD$^{br}$ cells in conventional and CryoStor media.
Data are presented as mean of ALD$^{br}$ percentage or recovery ± SD.

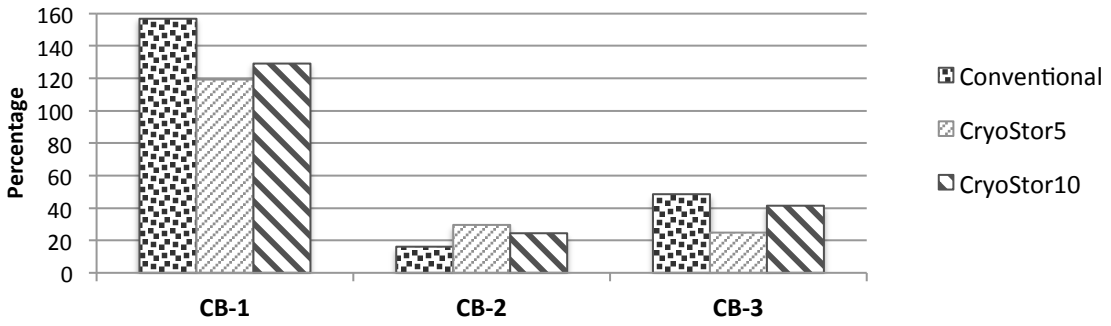



**Figure 13: Post-thaw recovery of ALDEFLUOR bright cells.**
Recovery of $ALD^{br}$ cells from each sample.

## Clonogenic capacity

To determine the clonogenic capacity of post-thawed samples, cells were inoculated in methylcellulose media and evaluated for the number of BFU-E, CFU-GM and CFU-GEMM colonies that grew for every $10^5$ cells plated. Results are shown in Table 8 and in Figure 14. Whether analyzing total colony count or each individual colony type, more colonies grew after cryopreservation in CryoStor 10 than in either of the other two cryopreservation medias, although the differences were not statistically significant.

**Table 8: Clonogenic capacity of post-thaw samples**

| | Conventional | CryoStor5 | CryoStor10 |
|---|---|---|---|
| **BFU-E** | 68 ± 41 | 72 ± 33 | 82 ± 38 |
| **P-value** | | 0.9 | 0.7 |
| **CFU-GM** | 53 ± 5 | 49 ± 14 | 58 ± 9 |
| **P-value** | | 0.7 | 0.5 |
| **CFU-GEMM** | 3 ± 1 | 2 ± 2 | 3 ± 2 |
| **P-value** | | 0.5 | 0.6 |
| **Total** | 124 ± 44 | 124 ± 44 | 144 ± 46 |
| **P-value** | | 1.0 | 0.6 |

Data presented as the mean number of colonies counted /$10^5$ cells plated ± SD. P-values compared the conventional with CryoStor media (N=3).

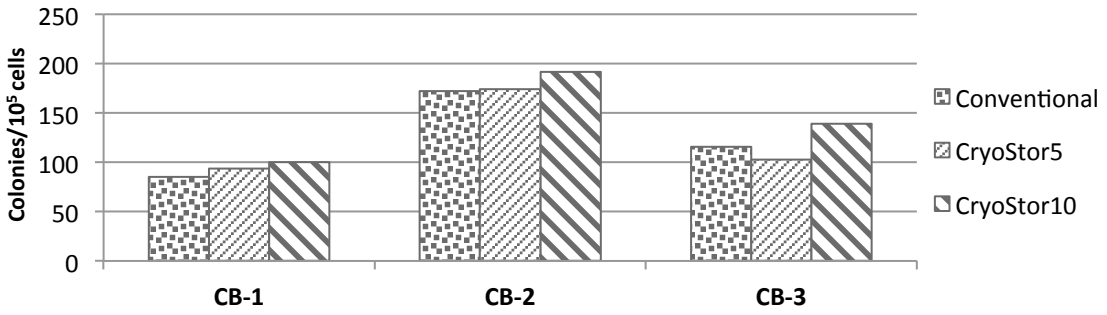



**Figure 14:Clonogenic capacity of post-thawed samples from each unit.**

### Proliferation capacity

Proliferation capacity was determined by inoculating thawed cells in StemSpan® SFEM serum free medium supplemented with a cytokine cocktail designed to support hematopoietic progenitor cell proliferation. After 48 h incubation, cells were washed and analyzed for proliferation capacity using a BrdU incorporation assay. Cell cycle stages were determined using the BrdU-FITC and 7-AAD plots. Evaluation was based on the percentage of CD38+ (CD45+, CD34+ and CD38+) that proliferated (were in either S phase,$G_2$, or M) or remained quiescent (in phase $G_0$ or $G_1$). The CD38+ progenitor cells cryopreserved with CS10 seemed better able to proliferate after thawing than if frozen in either CS5 or the conventional media, since there were more cells in either S or $G_2$/M phases of the cell cycle after 48 hrs in culture, however this difference was not statistically significant (p-value 0.07 – 0.8). Results are shown in Table 9 and Figure 15.

**Table 9: Proliferation potential of post-thaw samples**

| | Conventional | CryoStor5 | CryoStor10 |
|---|---|---|---|
| **S+G2/M %** | 22 ±12 | 25 ± 16 | 31 ± 18 |
| **P-value** | | 0.8 | 0.8 |
| **G0/G1 %** | 67 ± 12 | 66 ± 16 | 61 ± 17 |
| **P-value** | | 0.7 | 0.07 |

Comparison of CD38+ cells percentage in cell cycle stages (S+G2/M and G0/G1) for samples frozen in different cryopreservation media. P-values compared the conventional with CryoStor media. Data are presented as mean ± SD (N=3).

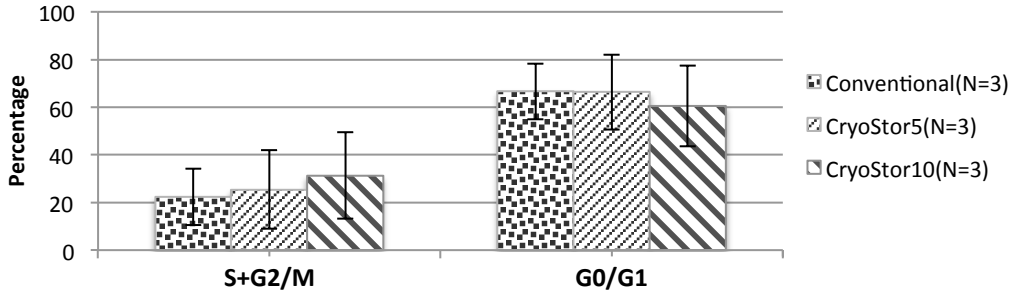



**Figure 15: Proliferation potential of post-thaw samples.**
Percentage of CD38+ cells at different cell cycle stages (S+$G_2$/M and $G_0$/$G_1$) after 48hrs in culture post-thaw. Data are presented as the mean ± SD.

## Hypothermal storage

Red blood cell depleted CBU were suspended in Plasmalyte/HSA or Hypothermosol (HTS-FRS® media) and stored in a 2-8$^{o}$C refrigerator. Samples were taken at 24, 48, and 72hr for analysis of cell subset viability, recovery, clonogenic capacity and proliferation ability.

Cell subset concentrations varied between CBU. For one of the CBU, CD90+ and CD90- concentrations were very low (0.001 % and 0.02 %). Because of that, that unit (CB-4) was excluded from the evaluation of these subsets.

### Viability of progenitor cell subsets

The viability (% 7-AAD neg, Annexin-V neg) of the various progenitor cell subsets after cold storage in two different transport media is shown in Table 10 and Figure 16. Viability of the CD45+ WBC population declined from an average of 75% at time 0 to an average of 62% after 72 hrs in HTS-FRS® media and to an average of 34% after 72 hrs in Plasmalyte /HSA, thus the viability declined to a significantly greater degree when stored in Plasmalyte /HSA than when stored in HTS-FRS® media (p-value 0.001). Similarly, the viability of the CD34+ cells declined more rapidly with storage in Plasmalyte/HSA, than they did during storage in HTS-FRS® media, but the difference was not statistically significant. The viability of the

other subsets analyzed either did not decline after 72 hrs, or the decline in viability was comparable for both storage medias.

**Table 10: Evaluation of cell subset viability in fresh and hypothermal stored samples.**

| | | CD90+ | CD90- | CD38- | CD38+ | CD34+ | WBC | CD3+ |
|---|---|---|---|---|---|---|---|---|
| **Day 0 Fresh** | %Viable | 88 ± 8 | 86 ± 5 | 97 ± 3 | 93 ± 3 | 94 ± 3 | 75 ± 9 | 94 ± 5 |
| **Day 1 Plasma-Lyte A/HSA** | %Viable | 91 ± 0 | 86 ± 0 | 96 ± 4 | 94 ± 2 | 95 ± 1 | 66 ± 2 | 94 ±6 |
| **Day 1 HTS-FRS®** | %Viable | 95 ± 0 | 87 ± 0 | 90 ± 6 | 94 ± 3 | 93 ± 1 | 66 ± 2 | 93 ± 2 |
| | P-value | 0.2 | 0.3 | 0.4 | 0.9 | 0.07 | 1.0 | 0.8 |
| **Day 2 Plasma-Lyte A/HSA** | %Viable | 92 ± 1 | 90 ± 1 | 89 ±10 | 91 ± 5 | 90 ± 6 | 58 ± 1 | 87 ± 7 |
| **Day 2 HTS-FRS®** | %Viable | 94 ± 1 | 93 ± 1 | 92 ± 3 | 92 ± 3 | 93 ± 1 | 60 ± 10 | 78 ± 17 |
| | P-value | 0.4 | 0.3 | 0.6 | 0.6 | 0.3 | 0.7 | 0.2 |
| **Day 3 Plasma-Lyte A/HSA** | %Viable | 93 ± 0 | 86 ± 0 | 85 ± 4 | 89 ± 4 | 82 ± 8 | 34 ± 2 | 88 ± 15 |
| **Day 3 HTS-FRS®** | %Viable | 91 ± 0 | 86 ± 0 | 96 ± 3 | 91 ± 2 | 94 ± 2 | 62 ± 13 | 88 ± 9 |
| | P-value | 0.7 | 0.8 | 0.1 | 0.5 | 0.2 | 0.008 | 1.0 |

P-values compare samples stored with conventional medium (Plasma-Lyte A/0.5%HSA) or HTS-FRS ® medium processed on the same day. Day 0, Day 1 and Day 2 (N=3); Day 3 (N=2).

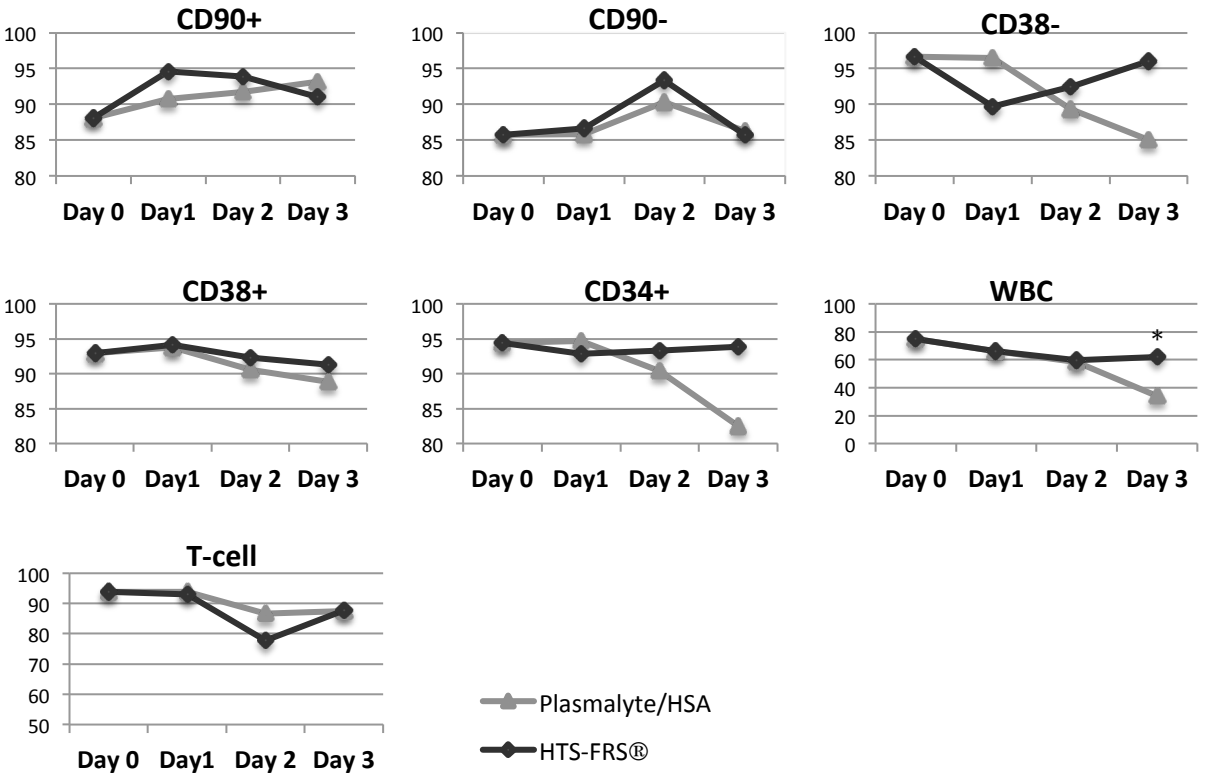



**Figure 16: Viability evaluation after hypothermal-storage.**
Data are presented as percentage mean. Day 0, Day 1 and Day 2 (N=3); Day 3 (N=2).

Similarly, there was not a statistically significant difference in the percentage of cells undergoing apoptosis after storage in either media, regardless of the cell subset analyzed (Table 11; Figure 17). The percentage of cells undergoing apoptosis was highest in the CD45+ WBC population, most likely due to the death of granulocytes during storage, however there was very little apoptosis occurring in any of the progenitor cell populations.

**Table 11: Apoptotic cell percentage in fresh and hypothermal-stored samples.**

| | | CD90+ | CD90- | CD38- | CD38+ | CD34+ | WBC | T CELL |
|---|---|---|---|---|---|---|---|---|
| **Day 0 Fresh** | %Apo | 8 ± 5 | 12 ± 5 | 3 ± 3 | 6 ± 5 | 5 ± 4 | 20 ± 8 | 6 ± 5 |
| **Day 1 Plasma-Lyte A/HSA** | %Apo | 7 ± 0 | 11 ± 0 | 3 ± 1 | 5 ± 3 | 4 ± 1 | 22 ± 1 | 6 ± 7 |
| **Day 1 HTS-FRS®** | %Apo | 4 ± 0 | 9 ± 0 | 9 ± 5 | 5 ± 2 | 6 ± 1 | 23 ± 3 | 7 ± 2 |
| | P-value | 0.3 | 0.3 | 0.3 | 0.8 | 0.5 | 0.5 | 0.9 |
| **Day 2 Plasma-Lyte A/HSA** | %Apo | 5 ± 0.5 | 7 ± 1 | 9 ± 9 | 7 ± 3 | 7 ± 4 | 16 ± 3 | 13 ± 7 |
| **Day 2HTS-FRS®** | %Apo | 4 ± 0.3 | 4 ± 0 | 7 ± 3 | 6 ± 3 | 6 ± 1 | 19 ± 5 | 22 ± 17 |
| | P-value | 0.2 | 0.6 | 0.7 | 0.6 | 0.4 | 0.2 | 0.5 |
| **Day 3 Plasma-Lyte A/HSA** | %Apo | 5 ± 0 | 6 ± 0 | 5 ± 5 | 5 ± 4 | 5 ± 4 | 13 ± 15 | 10 ± 12 |
| **Day 3 HTS-FRS®** | %Apo | 9 ± 0 | 8 ± 0 | 3 ± 3 | 7 ± 3 | 5 ± 2 | 15 ± 11 | 12 ± 9 |
| | P-value | 0.6 | 0.2 | 0.7 | 0.6 | 0.8 | 0.8 | 0.9 |

P-values compare samples stored with conventional medium (Plasma-Lyte A/HSA) with others stored with HTS-FRS® medium processed on the same day. Data are presented as percentage mean ± SD. Day 0, Day 1 and Day 2 (N=3); Day 3 (N=2).

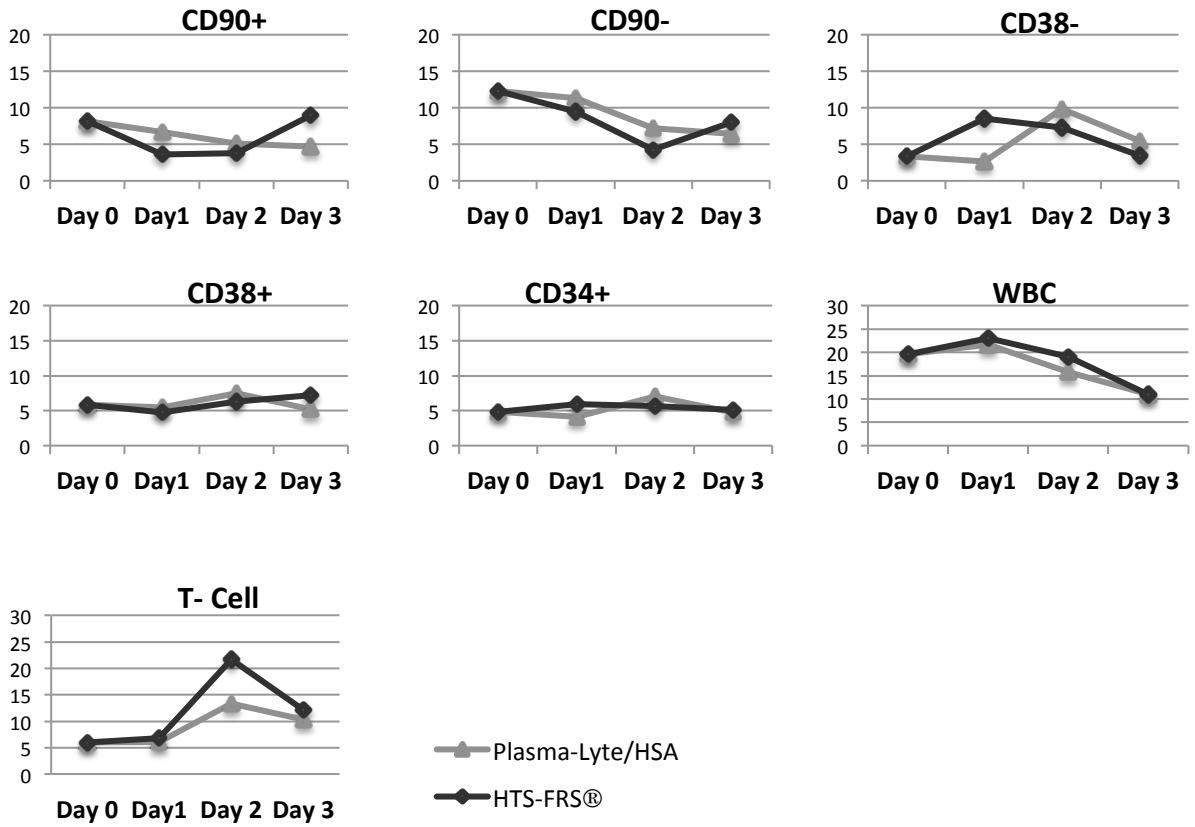



**Figure 17: Apoptotic populations after hypothermal-storage.**
Data are presented as percentage mean. Day 0, Day 1 and Day 2 (N=3); Day 3 (N=2)

Post-thaw TNC viable cell recovery varies between different cell subset. Variation between hypothermal-storage media was not statistically significant for HSC/P populations (CD90+, CD90-, CD38-, CD38+ and CD34+). On the other hand, variations in recovery of viable WBC and CD3+ after 72 h were statistically significant (p-value 0.001 and 0.01 respectively). Viable cell recovery increased for some cell subsets. Statistical study presented in Table 12. Figure 18 is a graphical representation of hypothermal stored cells recovery.

**Table 12: Recovery of cell subsets after hypothermal storage.**

| | | CD90+ | CD90- | CD38- | CD38+ | CD34+ | WBC | T CELL |
|---|---|---|---|---|---|---|---|---|
| **Day 1 Plasma-Lyte A/HSA** | Viable cell recovery % | 91 ± 31 | 97 ± 47 | 117 ± 26 | 139 ± 14 | 137 ± 22 | 87 ± 7 | 99 ± 27 |
| **Day 1 HTS-FRS®** | Viable cell recovery % | 113 ± 47 | 102 ± 51 | 94 ± 2 | 151 ± 13 | 137 ± 11 | 88 ± 6 | 96 ± 19 |
| | P-value | 0.7 | 0.9 | 0.2 | 0.4 | 1.0 | 0.7 | 0.9 |
| **Day 2 Plasma-Lyte A/HSA** | Viable cell recovery % | 165 ± 66 | 120 ± 76 | 195 ± 17 | 137 ± 6 | 146 ± 20 | 70 ± 5 | 81 ± 31 |
| **Day 2 HTS-FRS®** | Viable cell recovery % | 144 ± 27 | 102 ± 78 | 196 ± 13 | 127 ± 33 | 148 ± 30 | 66 ± 12 | 65 ± 39 |
| | P-value | 0.6 | 0.6 | 0.5 | 0.7 | 0.9 | 0.4 | 0.6 |
| **Day 3 Plasma-Lyte A/HSA** | Viable cell recovery % | 197 ± 0 | 92 ± 0 | 91 ± 4 | 106 ± 11 | 144 ± 78 | 29 ± 6 | 8 ± 3 |
| **Day 3 HTS-FRS®** | Viable cell recovery % | 182 ± 0 | 110 ± 0 | 148 ± 15 | 161 ± 18 | 146 ± 24 | 48 ± 2 | 49 ± 4 |
| | P-value | 0.3 | 0.7 | 0.1 | 0.4 | 1.0 | 0.001 | 0.01 |

P-values compare samples stored with conventional medium (Plasma-Lyte A/HSA) with others stored with HTS-FRS® medium processed on the same day. Data are expressed as percentage mean ± SD.

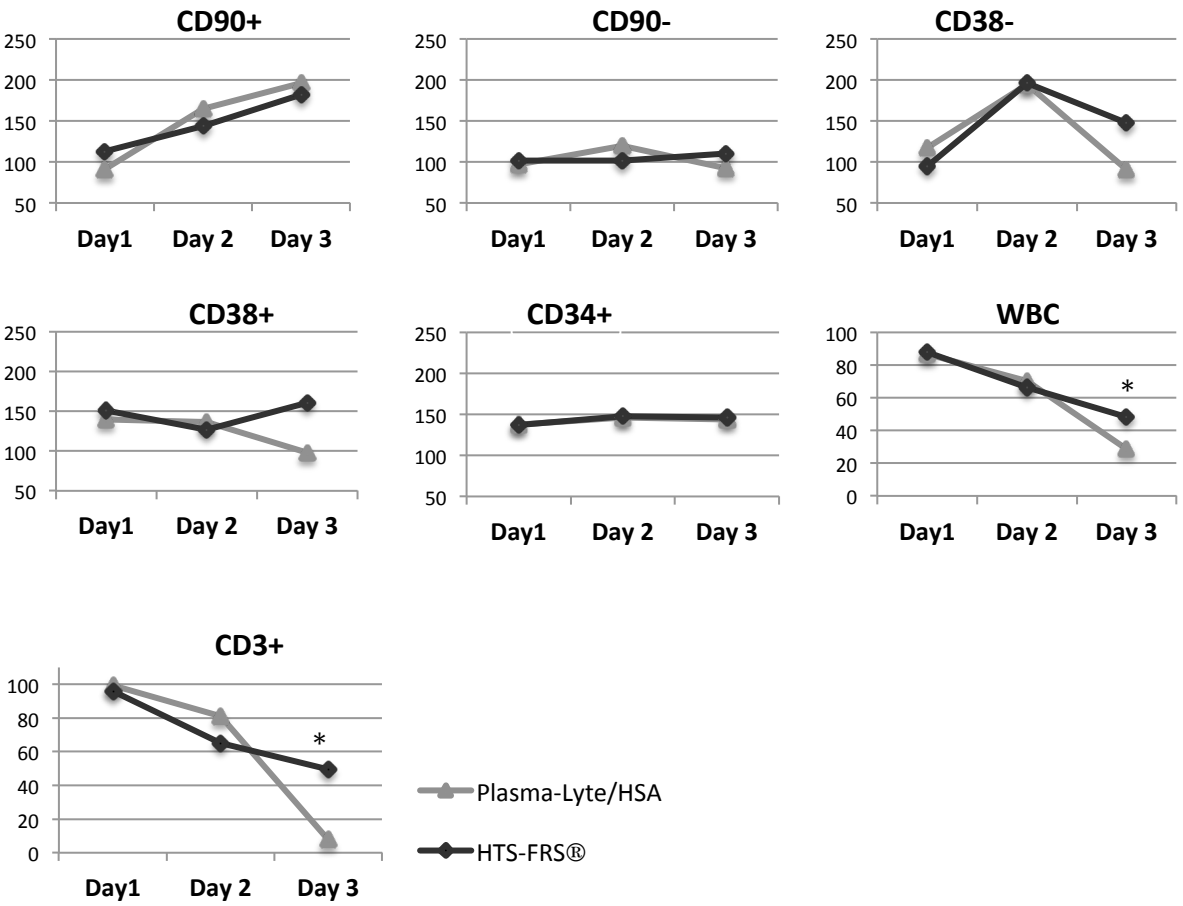



**Figure 18: Cell subsets recovery after hypothermal storage.**
Data are presented as percentage mean. Day 0, Day 1 and Day 2 (N=3); Day 3 (N=2).

**Recovery of ALDEFLUOR $^{Bright}$ cells**

Recovery of CD45+ CD34+, CD38- ALD$^{br}$ cells after storage for 48 or 72 hrs was significantly improved when the cells were stored in HTS-FRS®, compared to when the cells were stored in Plasmalyte/HSA. (p-values 0.01 and 0.02X10$^{-3}$ respectively). The difference was not significant after 24 hrs, however (Table 13; Figure 19).

**Table 13: Recovery of ALDEFLUOR$^{bright}$ cells after hypothermal-storage.**

| | % Recovery |
|---|---|
| **Day 1 Plasma-Lyte A/HSA** | 137.4 ± 70 |
| **Day 1 HTS-FRS®** | 161.1 ± 81 |
| **P-value** | 0.2 |
| **Day 2 Plasma-Lyte A/HSA** | 123.4 ± 93 |
| **Day 2 HTS-FRS®** | 181.4 ±62 |
| **P-value** | 0.01 |
| **Day 3 Plasma-Lyte A/HSA** | 48.3 ± 35 |
| **Day 3 HTS-FRS®** | 197.6 ± 90 |
| **P-value** | 0.00002 |

P-values compare samples stored with conventional medium (Plasma-Lyte A/HSA) with others stored with HTS-FRS® medium processed on the same day. Data are presented as percentage mean ± SD. Day 1 and Day 2 (N=3); Day 3 (N=2).

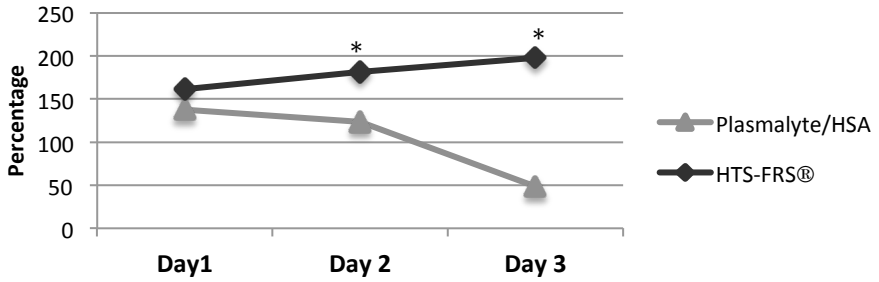



**Figure 19: Recovery of ALDEFLUOR bright cells after hypothermal storage.**
Data presented as mean of ALD$^{br}$ cells and recovery percentage. Day 1 and Day 2 (N=3); Day 3 (N=2).

**Clonogenic capacity**

Clonogenic capacity was evaluated after 48 and 72 h of refrigerated storage. The total number of colonies that grew per $10^5$ cells plated was reduced after 48 hrs (and 72hrs) of storage by approximately half (Table 14), however there was not a statistically significant difference in the recovery of clonogenic capacity between the 2 storage medias tested. The same pattern occurred for the individual colony types (CFU-GM; BFU-E; CFU-GEMM) as for the total number of colonies that grew after storage (Table 15). Also, the results were similar for each individual cord blood tested (Figure 20).

**Table 14: Clonogenic capacity after hypothermal-storage.**

| | Day 0 FRESH | DAY 2 Plasma-Lyte A/HSA | DAY 2 HTS-FRS® | DAY 3 Plasma-Lyte A/HSA | DAY 3 HTS-FRS® |
|---|---|---|---|---|---|
| **BFU-E** | 90 ± 37 | 63 ± 41 | 48 ± 26 | 40 ± 37 | 38 ± 32 |
| **P-value** | | | 0.6 | | 0.9 |
| **CFU-GM** | 95 ± 67 | 66 ± 53 | 49 ± 37 | 46 ± 51 | 40 ± 45 |
| **P-value** | | | 0.7 | | 0.9 |
| **CFU-GEMM** | 5 ± 1 | 1 ± 0 | 2 ± 2 | 1 ± 1 | 1 ± 1 |
| **P-value** | | | 0.6 | | 1.0 |
| **Total** | 190 ± 104 | 130 ± 94 | 99 ± 61 | 87 ± 88 | 79 ± 77 |
| **P-value** | | | 0.6 | | 0.9 |

Number of colonies /$10^5$ plated cells presented as mean ± SD (N=3)

**Table 15: Recovery of CFU**

| | DAY 2 Plasma-Lyte A/HSA | DAY 2 HTS-FRS® | DAY 3 Plasma-Lyte A/HSA | DAY 3 HTS-FRS® |
|---|---|---|---|---|
| **BFU-E recovery %** | 66 | 51 | 36 | 36 |
| **P-value** | | 0.3 | | 1.0 |
| **CFU-GM recovery %** | 65 | 52 | 34 | 30 |
| **P-value** | | 0.1 | | 0.9 |
| **CFU-GEMM recovery %** | 23 | 31 | 16 | 16 |
| **P-value** | | 0.7 | | 1.0 |
| **Total recovery %** | 63 | 51 | 35 | 33 |
| **P-value** | | 0.3 | | 0.9 |

Recovery of CFU in hypothermal stored samples. P-value calculated comparing the recovery of samples stored with Plasma-Lyte A/0.5 % HSA vs. HTS-FRS® (N=3).

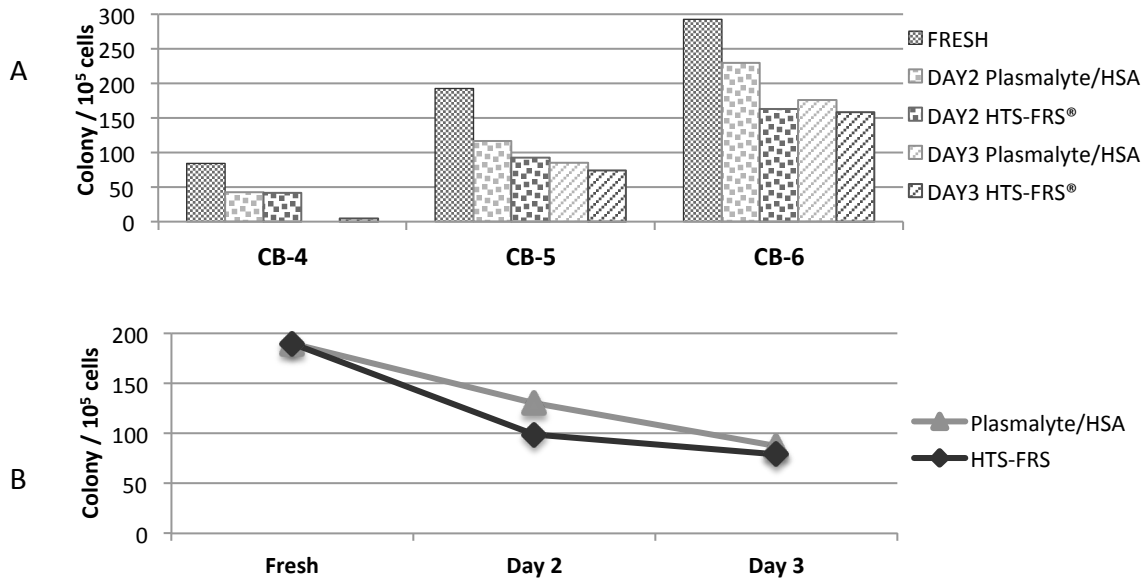



**Figure 20: Clonogenic capacity after hypothermal storage.**
(A) Total number of formed colonies /$10^5$ plated cells for each unit. (B) Mean of the total number of formed colonies /$10^5$ from the three samples.

**Proliferation capacity**

The ability of the CD34+, CD38- progenitor cells within the cord blood samples to proliferate in culture in response to cytokine stimulation was tested after 48 and 72 h of refrigerated storage using the same procedures followed for post-thaw samples. A slightly larger percentage of the cells were able to proliferate in culture after storage in HTS-FRS® compared to Plasmalyte/HSA, however this difference was not statistically significant (p-value 0.5 - 0.9) (Table 16; Figure 21-A and B). Interestingly, comparing the results shown in Table 16 with those shown in Table 9, a larger percentage of the cells were able to proliferate in culture (higher G2+M percentage) after the cells were cryopreserved and thawed than after 48 hrs of refrigerated storage.

**Table 16: Proliferation potential after hypothermal-storage.**

| | S+G2/M | G0/G1 |
|---|---|---|
| **Day 2 Plasma-Lyte A/HSA** | 12.1 ± 6 | 13.5 ± 10 |
| **Day 2 HTS-FRS®** | 17.2 ± 2 | 11.1 ± 5 |
| **P-value** | 0.8 | 0.9 |
| **Day 3 Plasma-Lyte A/HSA** | 17.3 ± 3 | 9.1 ± 6 |
| **Day 3 HTS-FRS®** | 20.3 ± 9 | 9.1 ± 4 |
| **P-value** | 0.5 | 1.0 |

Comparison of CD38- cells percentage in proliferative staged (S+G2/M), quiescent stages (G0/G1) for samples stored at different hypothermal storage media. Data are presented as percentage mean ± SD (N=3).

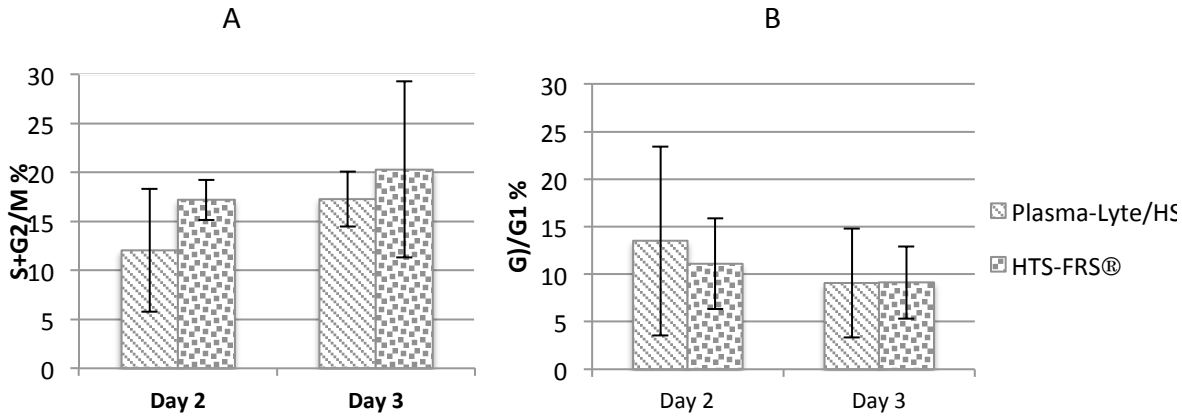



**Figure 21: BrdU incorporation assay evaluation after hypothermal-storage.**
(A) Percentage of cells in proliferative stages S+G2/M. (B) Percentage of cells at quiescence stages (G0/G1)(N=3).

## DISCUSSION

In this study, a comparison was made between commercially available serum free cryopreservation solutions (CryoStor®) that contain either 5 % or 10 % DMSO with Hoxworth Blood Center's conventional formulation, containing 5 % DMSO, 2.5 % HES, 10 % ACD-A and 10 % HSA. Comparison was based on post-thaw viability and recovery of viable cell subsets (CD90+, CD90-, $ALD^{br}$, CD38-, CD38+, CD34+, WBC and CD3+), clonogenic capacity, and proliferation potential. The results showed a variation in cell viability and viable cell recovery between different cell subsets in different cryopreservation media. Recovery of progenitor cells subsets (CD38-, CD38+, and CD34+) was better than it for the overall recovery of WBC (CD45+), which is consistent with previous findings that CD34+ cells are more resistant to cryopreservation than other mononuclear cells, perhaps because of their quiescent nature or because of the nature of their membrane lipid and protein content.[37-39] The concentration of CD3+ cells was low in fresh CBU (23 %) and it decreased sharply in post-thaw samples (10, 7 and 13 % in conventional, CS5, and CS10 respectively). That decrease resulted in a poor recovery of the CD3+ population (37±9 %, 27 ± 15 % and 51 ± 10 % in conventional, CS5, and CS10 respectively). Stroncek et al. evaluated CD3+ recovery in post-thaw WBC collected from HSC transplant donors (N=311); recovery of CD3+ was 76 ± 16 %.[40] On the other hand, viability of WBC decreased sharply in post-thaw samples resulting in a poor recovery of viable WBC with all cryopreservation media. That decrease might be caused by the loss of granulocytes in post-thaw samples (Figure 22). Evaluation of the total WBC viability was included in the study to emphasize differences between storage media, despite the fact that loss of the mature granulocyte will not affect the quality of HSC products because these cells don't play a role in short or long term engraftment.

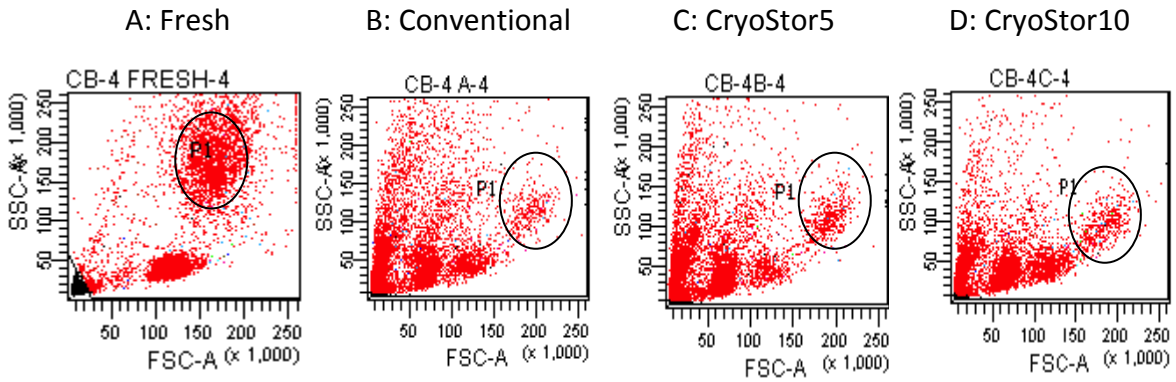



**Figure 22: Loss of granulocytes in post-thaw samples.**
Flow cytometry light scatter plots show a significant loss of the granulocyte population in post-thaw samples. FSC-A = forward scatter area. SSC-A = side scatter area.

Other cell HSC/P subsets were not included in a comparison of cryopreservation media in previous studies. In comparing cryopreservation media, there were no significant differences in the viability or recovery of all cell subsets. Results showed a slight variation in clonogenic capacity and proliferation potential between samples stored with different cryopreservation solutions with preferable results for cells cryopreserved with CS10. There was a strong positive correlation between proliferative cells and CFU in CB1 and CB-2 (R=1) and negative correlation with CB-3 (R=-0.9).

Variation between cryopreservation media in viability, recovery of viable cell subsets and in-vitro functional assays were not statistically significant.

Other studies have been performed in the past to evaluate the effect of different DMSO concentrations on cryopreservation of HSC. Liseth et al. used PBPC-derived stem cells to determine the differences in viability (7-AAD only) of CD34+ and WBC in post-thaw samples using different DMSO concentrations. DMSO was added directly to HSC suspension (with autologous plasma). This study concluded that cell concentration at cryopreservation had an effect on the post-thaw result and that 5 % DMSO is the optimal concentration for

cryopreserving PBPC, if the cell concentration did not exceed 200 X $10^6$ NC/mL.[41] Variation in the duration of liquid nitrogen storage proved to have no effect on the recovery or viability post-thaw, however the procedures for cryopreservation, thawing, cell subset identification, viability evaluation and timing before testing differed from our study, making comparisons difficult.

Abrahamsen et al. also used PBPC derived stem cells to compare viability (7-AAD and annexin-V) of post-thawed samples stored in 5 % or 10 % DMS0. They followed the same cryopreservation procedure that Liseth et al. used. Their results indicated that 5 % DMSO resulted in better viability and recovery of CD34+ cells. Furthermore, 5 % DMSO resulted in less Apoptosis (7-AAD neg.; annexin-V pos.) and necrosis (7-AAD pos.; annexin-V pos. or neg.), however their cryopreservation evaluation did not include any functional measurement of the recovered post-thaw cells. [42]

Other studies comparing the commercially available CryoStor media with other lab-formulated media concluded different findings. Clarke et al. used peripheral blood HSC/P cells at 50 X $10^6$/mL to compare CS5, CS10 and a conventional media formulation used at Fred Hutchinson Cancer Research Center (FHCRC) that contains Normosol-R, 5 % HSA and 10 % DMSO. Cryopreservation media were added in 1: 1 dilution then samples were thawed and diluted to 1:32 in TM containing D-PBS and 11 % FBS. Evaluation was based on recovery and viability of TNC, CD34+ and granulocytes (using 7-AAD only), and CFU (N=9). Results revealed that samples stored in CS10 yielded a significant increase in recovery of viable CD34+ and CFU. [43] Another study by Stylianou et al. used CB derived HSC to compare CS5, CS10 with 10 % DMSO in dextran-40. Evaluation was based on TNC, CFU and recovery and viability of CD34+ (using annexin-V and 7-AAD) (N=30). Units were thawed and mixed for 2

min. before testing. Post-thaw results from samples stored with CS10 showed an improvement in TNC recovery, clonogenic capacity and CD34+ recovery and viability. Improvement in CD34+ recovery and viability was not statistically significant with CS5. [31] These findings are similar to our results; the samples cryopreserved with CS10 showed a trend to produce more colonies but it was not statistically significant (p-value 0.6 for the total number of formed colonies/$10^5$ plated cells).

Comparing the conventional and HTS-FRS® showed similar viability, viable cell and $ALD^{br}$ recovery results after 24 h (day 1) and the variation increases after 24 h of storage with a preferable viability, viable cell recovery and proliferation capacity from samples stored with HTS-FRS®. That variation was statistically significant for viable WBC at day 3, recovery of viable WBC and CD3+ at Day 3 and recovery of $ALD^{br}$ at day 2 and 3, with superior results from samples stored in HTS-FRS®. Furthermore, an increase in the viability of the CD38- population from samples stored in HTS-FRS® was associated with an increase in the $ALD^{br}$ recovery and an increase in the recovery of viable CD38+ was associated with an increase in the proliferation potential. The clonogenic capacity decreased in hypothermal stored samples with both media with better recovery for samples stored with Plasma-Lyte A/0.5 HSA.

Mathew et al. have shown that storing different kinds of tissues in HTS-FRS® at 4°C improves post-storage viability and functionality. They also suggested that preservation solutions should be designed to meet the unique molecular and biological requirements for each cell type. [29] A better understanding of the molecular biology of HSC apoptosis could lead to improved storage by modifying the ingredients of the HTS-FRS® to reduce cell loss.

Correlation between the viable HSC/P cell subsets (CD90+, CD90-, CD38-, $ALD^{br}$, CD38+ and CD34+) and the CFU and proliferation (G2/M stage) data was not possible. That necessitates an in-vivo study to know the effect of viability percentage of HSC/P subset in its functionality (engraftment outcomes).

Commercially available media CryoStor® and HTS-FRS® contain similar components except for the DMSO. HTS-FRS® contains more nutrition which provides stored cells with the required support when its metabolic activities are reduced and more pH stabilizer to overcome changes in the pH during storage. The HTS-FRS® has an advantage over the Plasma-Lyte A/0.5% HSA as it provides more nutrition, better pH control, free radical scavengers. Perhaps an improvement in the conventional hypothermal storage media (Plasma-Lyte A/0.5% HSA) could be achieved by increasing the albumin concentration (2 g/dL) to mimic its normal physiological levels in the plasma (3.4-5.4 g/dL).

## CONCLUSIONS

Our results indicate that cell subsets differ in their resistance to cryopreservation injury, as indicated by differences in post-thaw viability measurements. Comparing the conventional cryopreservation medium with CS5 and CS10 showed no statistically significant difference between the groups, but with a trend toward improvement in clonogenic capacity and proliferation potential with CS10. Further evaluation with more samples is required to support the benefits of CS10 over the conventional media. In general, as the low concentration of DMSO reduces the DMSO toxicity upon transfusion, and the variation between conventional and CS5 was not statistically significant, the conventional medium is equally effective in cryopreservation of HSC products.

The hypothermal storage study proved that the conventional (Plasma-Lyte A/0.5 %HSA) medium is suitable for holding HSC products for 24 h, but not for transporting fresh products if the transport duration will be more than 24 h

Using the commercially available CryoStor® or HTS-FRS® media rather than preparing it in the lab is beneficial as it will standardize the cryopreservation procedures and reduce errors in processing cellular therapy products caused by the multistep conventional processes. Increasing the sample size, including other cell types, and performing in-vivo analyses might enable the detection of minor differences in these storage media.

## REFERENCES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